Chromatographic studies on the detection of some basic drugs of abuse

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CHROMATOGRAPHIC STUDIES ON THE DETECTION OF SOME BASIC DRUGS OF ABUSE

Thesis

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DEDICATION

To my children, mother and the memory of my late Father.

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SUMMARY

1. Two approaches have been studied to enhance the selectivity of chromatographic systems suitable for basic drugs of abuse. The behaviour of nineteen compounds has been studied when chromatographed using column materials that are low in carbon loading. This has permitted the selection of mobile phases that are low in organic modifier, which has minimised background UV absorption at 205 nm. Three silica-based column materials, namely cyanopropyl-, Hypersil SAS and Nucleosil SA gave good selectivity. The cyanopropyl- material (Hypersil or Spherisorb) is recommended due to its shorter analytical times and better stability. In general, solutes may be detected down to 2-5 ng on-column.
2. The behaviour of seven compounds has been studied using a post-column extraction detection system, using naphthalene-2-sulphonate as a UV-indicating pairing-ion. The design of the system was optimised for maximum selectivity, and included testing a novel phase separator capable of delivering a very high percentage of organic extraction phase to the detector. In general, solutes may be detected down to 20-100 ng on-column.
3. Both methods gave linear calibrations and good recovery levels for spiked plasma.

4. The influence of temperature and mobile phase organic modifier content upon analyte retention has been rationalised by their influence upon analyte pKa and log P values. This was achieved by the determination of pKa and log P values for nineteen compounds in chromatographic mobile phases, and other solutions related to them. The results indicate that literature pKa values for organic amines should be used with caution and should in any case be reduced by 0.027 per °C rise in mobile phase temperature.

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SECTION 1

INTRODUCTION

SECTION 1 INTRODUCTION

1.1 DRUGS OF ABUSE

The abuse of drugs generally involves the self administration of a drug in a manner and with an effect that is not approved of by the medical profession and the general public. It is a sociological and medical problem that continues to expand every year, and one that is increasingly difficult to eradicate. One essential aspect of this problem is the development of analytical techniques that may be used to identify and quantify drugs of abuse, whether as pure drugs, as formulated medicines, or as trace amounts present in biological fluids such as blood and urine. These analytical techniques provide the evidence that is required in order to control the illegal possession and misuse of drugs. These techniques need to be as simple and reliable as possible and applicable to as many drugs as possible so as to minimise the number of analytical methods required. Gas chromatography (GC) employing packed columns has been extensively used for a wide range of drugs, including drugs of abuse for many years (1). More recently, the superior column efficiency of capillary columns have been appreciated, especially when linked to a mass spectrometer that is able to provide peak identification at high levels of sensitivity. However this technique is still very

expensive and cannot yet be regarded as widely available as a routine instrument, although mass spectrometers specifically designed as GC detectors are much less expensive than high resolution instruments.

Over the past ten years, High Performance Liquid Chromatography (HPLC) has developed into a highly flexible sophisticated and sensitive analytical technique that can be regarded as routinely available in analytical laboratories. The majority of medicinal compounds have a solubility in water that is adequate to permit their analysis using aqueous-organic solvent mixtures with reversed-phase columns. Additionally, most compounds exhibit sufficient absorbance in ultraviolet radiation to permit their detection by a UV spectrophotometric detector. However, adequate detection is very dependent upon the sensitivity required to meet the analytical need, so that there are many examples of drugs that cannot be adequately detected at the levels commonly encountered in plasma or urine.

We were aware (2) that a considerable number of basic drugs possessed weak UV absorbance, that is having molar absorptivities in the region of 200 or less. At this level of absorption, compounds are required to be present at about 1 $mgml^{-1}$ level in order to detect the signal from a 20 µl injection, i.e. 20 µg of drug. Anticipated levels in plasma, except in the

case of massive overdose, would normally be in the low $ngml^{-1}$ range, i.e. 10^{-6} lower than the solution required for UV detection. We were advised by the Drugs and Toxicology Division of the Home Office Central Research Establishment, Aldermaston, that there were a number of drugs of this type covered by the Misuse of Drugs Act (1971) which could serve as model compounds for a study into methods designed to improve sensitivity of detection (3). They were all drugs of obvious forensic interest and included amphetamine, benzphetamine, chlorphentermine, fentanyl, mephentermine, methylamphetamine, pethidine, phenmetrazine, phendimetrazine, pipradrol and methadone. Because many of these compound are tertiary amines, chemical derivatization to enhance UV absorption or fluorescence is not generally possible. In these circumstances, an increase in equipment complexity is justified, and the main approach was to study post-column derivatization using UV-sensitive ion-pair reagents. This approach required an extraction step using an immiscible organic solvent which meant that a phase separator would be needed, to cleanly separate the organic phase from the aqueous phase prior to passing the organic phase through the detector. Preliminary studies had already been made (4) with a separator made to a design by Kinkel and Tomlinson (5) that appeared promising for this application. If

successful, a range of chemical derivatization reagents could be studied to assess their suitability for routine use. The drugs selected for this study were chosen on the basis of their importance and availability, as indicated in the 28th Edition of Martindale (6). The most important group contains drugs listed as being currently available, and were amphetamine and its metabolite hydroxyamphetamine, diphenoxylate, dipipanone, fentanyl, methadone, methylamphetamine, normethadone, norpipanone, pethidine and piritramide. A second group consisted of drugs now discontinued in the UK but perhaps still available overseas, and were benzphetamine, chlorphentermine, mephentermine, phenmetrazine, phendimetrazine and pipradrol. Phenylethylamine was included as a possible internal standard for the amphetamine series.

A third group of drugs suggested for study were suitable analytically, but were not listed in Martindale, and it was considered that future supplies would be difficult to obtain. They were allylprodine, alphacetylmethadol, alphamethadol, benzethidine, diampromide, dioxaphetylbutyrate, noracymethadol, phenampromide, properidine and racemoramide. This group was excluded from the study.

The nineteen compounds examined in this study are stimulant or narcotic primary, secondary or tertiary amines, whose UV absorbance at 254 nm is inadequate to

make their detection possible at the $ngml^{-1}$ range. An appreciation of their physico-chemical properties is needed in order to propose satisfactory chromatographic procedures, and this now follows.

1.2 PHYSICO-CHEMICAL PROPERTIES

1.2.1 Sympathomimetic Amines

Dexamphetamine sulphate

Synonym: dextroamphetamine sulphate (+)-α-methylphenethylamine sulphate $(C_9H_{13}N)_2H_2SO_4$. mol. wt. = 368.4.

This is a white, odourless crystalline powder with melting point above 300° pH 5-6 (1 in 20 solution), specific rotation (4% aqueous solution of a dried sample) is between 20° and 23.5°. Soluble 1 in 9 water and 1 in 800 ethanol, but insoluble in ether. Its dissociation constant (pKa) is 9.9 at 20°C. Dexamphetamine is unusual in being a (+)-isomer which is more active pharmacologically than the corresponding (-)-isomer (1, 7-10).

Hydroxyamphetamine sulphate

A white crystalline powder, soluble 1 in 1 of water and 1 in 2.5 of ethanol. It is slightly soluble in chloroform and almost insoluble in ether. An aqueous solution is slightly acidic to litmus (pH about 5), and gives a purple colour with ferric chloride solution.

Melting point from 189° to 192°C (6-8, 11).

Phenylethylamine

it is a strongly basic liquid which absorbs carbon dioxide from the air. It has a boiling point of 194.5°C - 195°C. It is soluble in water, freely soluble in ethanol and ether (7).

Methylamphetamine sulphate

Methylamphetamine is considered to be the N-methyl derivative of amphetamines or as a deoxy derivative of ephedrine, with a molecular weight of 245.24. The sulphate salt is an odourless, white crystalline powder with a bitter taste. A 1% solution is neutral or slightly acidic to litmus. It is soluble in water, alcohol and chloroform, but insoluble in ether and acetone. Its melting point range is 171-175°C and a 2% solution of it has an optical rotation of +16° to +18° (1, 6-11).

Mephentermine sulphate

Mephentermine sulphate is N-α,α-trimethyl-phenethylamine sulphate dihydrate and contains, on the dry basis not less than 98 percent of $(C_{11}H_{17}N)_2H_2SO_4$. It is an odourless crystal or crystalline powder soluble 1 in 20 of water and 1 in 150 of alcohol, insoluble in chloroform. A 2% solution in water has a

pH of 4-6.5 while 4.74% solution is iso-osmotic with serum (1, 6, 7, 10, 11).

Chlorphentermine hydrochloride

It is a white to off-white crystalline powder, odourless and with a bitter taste, melts at about 235°C. Freely soluble in water and alcohol, sparingly soluble in chloroform, practically insoluble in ether. The pKa is about 9.6.

Phendimetrazine bitartrate

A white crystalline powder, odourless, bitter taste, melts at about 186° with decomposition and has a pKa 7.2 It is soluble in water and alcohol.

Pharmetrazine theoclate

It is a white powder soluble in water and ethanol, slightly soluble in ether.

Benzphetamine hydrochloride

A white to off-white crystalline powder with melting point ranging from 131°-135°. It is soluble in water, alcohol and chloroform, slightly soluble in ether.

1.2.2 Narcotic Analgesics

Analgesics are agents which relieve pain by acting

centrally to elevate the pain threshold without disturbing consciousness or altering other sensory modalities. The most widely used powerful analgesic, morphine, has many undesirable side-effects and this has stimulated the development of synthetic alternatives (6, 7). However all these compounds retain some addictive effects, so that they are subject to abuse for their central nervous system effects and have become an analytical problem for forensic laboratories.

Fentanyl citrate

This is a white glistening crystalline powder or white granules, odourless and tasteless, it is stable in air and melts between 147°C and 152°C with a pKa of 7.3. Soluble 1 in 40 of water, sparingly soluble in ethanol, ether and chloroform (1, 6-9, 11).

Pethidine hydrochloride

A white crystalline powder with melting point betwen 187°-189°C. Very soluble in water, 1 in 20 of ethanol, sparingly soluble in ether, but soluble in chloroform. A 2% solution in water has a pH of 4.5-5.5 and a 4.8% solution is iso-osmotic with serum. It has a pKa of 8.7 (1, 6-11).

Pipradrol hydrochloride

It is an odourless, colourless crystal or a white

crystalline powder which melts at about 295° with decomposition. Soluble 1 in 30 of water, 1 in 35 of ethanol and 1 in 1000 of chloroform, almost insoluble in ether. It has a pKa of 9.71. A 1% solution in water has a pH of 5 to 7 (1, 6).

Piritramide

A crystalline powder with melting point of 149° to 150°C. Soluble in dilute acetic acid, slightly soluble in water (1, 6).

Methadone hydrochloride

Odourless, colourless crystals or crystalline powder with bitter taste. Melts between 233°-236°C. Soluble 1 in 12 of water, 1 in 7 of ethanol and 1 in 3 of chloroform, insoluble in ether. A 1% solution in water has a pH of 4.5 to 6.5 (1, 6-11).

Normethadone hydrochloride

Crystals with melting point 174°-177°, soluble in water and alcohol. A 1% solution in water has a pH of about 5 (1, 6).

Dipipanone hydrochloride

An almost colourless, white crystalline powder with a bitter numbing and burning taste. Melting point 124°-127°. Soluble 1 in 40 of water, 1 in 1.5 alcohol

and 1 in 6 of acetone, insoluble in ether. A 2.5% solution in water has a pH of 4-6 (1, 6-11).

Norpipanone hydrochloride

Crystals with melting point from 181° to 182°C. Soluble in water and alcohol (1, 6).

1.3 PHARMACOLOGICAL ACTIONS AND USES

Sympathomimetic amines are classified as a group of basic compounds which mimic the effects of stimulation of the sympathetic nerves to the organs of the body (11). The amphetamines may be considered together because of similar pharmacological properties which they possess. They are sympathomimetic compounds and the dominant feature of these drugs is their potent stimulatory action on the central nervous system (CNS), in addition to the peripheral α and β actions common to the sympathomimetic drugs (11, 12).

Some of the characteristics of this stimulating action on the CNS are facilitation of thoughts, improvement of physical performance, analepsis, elevation of fatigue levels, increased confidence, elation and euphoria. All these desirable effects are often followed by a "rebound" to a state of depression and fatigue. Peripherally, it produces vasoconstriction, contraction of the bladder, sphincter and relaxes the bronchial muscle. The amphetamines are

therapeutically said to have the following indications (11):

a) Hyperkinetic behavioural disorders in children with brain damage are calmed by controlled doses of amphetamines although they are used as long term treatment, paradoxically, tolerance is rare.

b) Dextroamphetamine has been used to control exogenous obesity more effectively than a racemic mixture, but it is usually not recommended for periods longer than 6-10 weeks to avoid tolerance.

c) Narcolepsy, in this case it controls the symptoms, but it is not curative. The dextro-isomer is more effective than the laevo-isomer, but there is the tendency of developing tolerance to the drug.

Usually, the term narcotic refers to a drug that diminishes sensibility, relieves pain and induces lethargy, drowsiness, or sleep. Narcotics produce indifference both to physical discomfort and to mental anguish. Chronic suppression of central nervous system function results in a dependent state in which the drug must be taken regularly.

Dexamphetamine

This is an indirectly acting sympathomimetic compound with weak peripheral but strong CNS stimulant actions. As a central stimulant, the dextro-rotatory isomer is about twice as potent as the racemic mixture

(amphetamine) and 3-4 times as potent as the laeveo-rotatory isomer. The actions and uses are as mentioned for sympathomimetics. As an anorectic agent, it has been supplanted by anorexiants with weaker euphoric actions, to which tolerance develops less rapidly. It has been used as a pressor agent in orthostatic hypotension and in spinal anesthesia.

Hydroxyamphetamine

Hydroxyamphetamine is an indirect sympathomimetic, the actions of which are both α and β agonist in nature, it is essentially devoid of central nervous activity. It is used topically as a nasal decongestant and together with atropine as a mydriatic agent. The para substituted hydroxy group markedly alters the pharmacodynamic properties of amphetamine. It is 2-4 times more active as a pressor agent therefore increasing blood pressure especially in spinal anesthesia and orthostatic hypotension by myocardial stimulation and peripheral vasoconstriction.

Methylamphetamine

This is a central stimulant with an analeptic effect greater than that of amphetamine. It had been used to antagonise barbiturate hypnosis, for the relief of mental and physical fatigue and counteract acute alcoholism. It acts directly on the α and β receptors and

is used in the management of hypotension associated with spinal anesthesia.

Mephentermine

It is a compound with strong α-adrenergic activity and is therefore used in the management of various states of hypotension and in the treatment of nasal congestion.

Chlorphentamine

It is generally recommended for use as an adjunct to lessen appetite in the management of obesity. There is not sufficient evidence to show that it has any primary effect on appetite-control centres of the hypothalamus. The anorexigenic effect is temporary. Chlorphentamine produces less stimulation of the central nervous system than other anorexiants of the amphetamine type.

Phendimetrazine

It is an indirectly acting sympathomimetic. It is used as an appetite suppressant.

Phenmetrazine

It is used as an anorexigenic agent, for which it is about equal to dextroamphetamine. Studies have indicated that phenmetrazine may have greater relative

central stimulatory activity than amphetamine.

Benzphetamine

It is only approved for use as an anorexiant (i.e. in the treatment of obesity), in which role it is about as efficient as dextroamphetamine.

Pipradrol

Pipradrol has been reported to be of use in the treatment of fatigue.

Fentanyl

Fentanyl is a potent narcotic analgesic with rapid onset and short duration of action. Its pharmacological actions are similar to morphine. Equal analgesia can be obtained with a dose 1/150 that of morphine.

It is used primarily as an analgesic for the control of pain associated with all types of surgery. It can also be used as a supplement to all agents commonly employed for general and regional anesthesia. It is also an ingredient in fentanyl citrate and droperidol injection.

Pethidine

Pethidine is less powerful than morphine but has considerbly shorter duration of action. Depending on the route of administration, the analgesic action of

pethidine is observed within 15 minutes reaching a peak in about an hour and subsiding in 2 to 4 hours. Since it reduces the sensitivity of labour pains without seriously diminishing the force of uterine contraction, pethidine is used as an obstetric analgesic, it may however prolong labour. It has also been substituted for morphine in both pre- operative and post-operative medication. It is sometimes used in association with local and spinal anaesthetics and to supplement nitrous oxide and oxygen anaesthesia.

Piritramide

It has analgesic uses similar to those of morphine and is discussed under narcotic analgesics.

Methadone

It is used as an analgesic to relieve moderate to severe pain, particularly in chronic conditions, pain in post-operative states is lessened by the drug. It is also used as an antitussive, suppressing the cough reflex, but such use may lead to addiction. The principal actions of therapeutic value are those of analgesia, sedation and detoxification or temporary maintenance in narcotic addiction.

Normethadone

It is used mainly as a cough suppressant, but its

action and general use are as discussed under narcotic analgesics.

Dipipanone

This is a potent analgesic related to methadone. It has relatively little sedative and hypnotic action compared with methadone. It can be used to maintain relief of pain when morphine or pethidine has ceased to be effective.

Norpipanone

It is used to relieve pain in smooth muscle spasm and migraine.

1.4 PHARMACEUTICAL FORMULATIONS

Dexamphetamine B.P.

(i) Capsules, tablets and sustained release forms containing from 2.5 to 20 mg of dexamphetamine sulphate alone or in combination with any of the following: methylamphetamine, amylobarbitone, phenobarbitone, aspirin, phenacetin, codeine trifluroperazine, meprobamate, methaqualone and vitamins.

(ii) Elixirs, containing 1 mg/ml of dexamphetamine sulphate alone or with amylobarbitone.

Recommended doses

a) Narcolepsy: Adults 1060 mg daily in divided doses.

b) Minimal brain dysfunction: children over 3 years old: 5-40 mg daily in divided doses.

c) Barbiturate poisoning: 10-50 mg/ml in a slow intravenous drip.

d) Exogenous obesity: Adults 5-10 mg taken 30 to 60 minutes before meals.

Hydroxyamphetamine

(i) Ophthalmic solution (1%) isotonic with tears.

(ii) Tablets: 20 mg

Recommended doses

a) Postural hypotension: 60-400 mg orally in divided doses.

b) Heart block: 20-60 mg three or four times a day.

c) Mydriatic: 1-2 drops of 1% ophthalmic solution.

d) Carotid sinus syndrome: 20-60 mg orally.

Methylamphetamine hydrochloride B.P.

Capsules or tablets containing from 2.5 to 10 mg alone or in combination with amphetamine or dexamphetamine sulphate, phenobarbitone or phenytoin.

Recommended doses

a) Analeptic: Adults: 15-60 mg orally or 30 mg I.V. or I.M.

b) Antidepressant: Adults 5-60 mg daily in divided

doses.

c) Anorexic: 2.5-5 mg before meals.

d) Pressor agent: 10-30 mg I.M. and 5-10 mg I.V. drip.

Mephentermine sulphate

(i) Mephentermine sulphate is available in several injection forms containing 15-30 mg/ml, tablets containing 12.5-25 mg.

(ii) Nasal drops: 0.5% solution.

Recommended doses

a) Treatment of shock: 20 mg I.M. or 30-60 I.V.

b) Treatment of hypotension associated with spinal anesthesia:

Adult: 30-45 mg I.M. or 12.5-25 mg adminsitered orally 1 or 2 times daily.

Children: 0.4 mg per kg administered orally, I.M. or slowly I.V.

Chlorphentermine hydrochloride

Tablets, 65 mg as chlorphentermine base.

Recommended doses

a) Anorexic: orally, user over 12 years of age only: 65 mg of the base once a day.

Phendimetrazine bitartrate

Tablets containing 35 mg.

Recommended doses

Anorexic: Adult: 17.5 - 70 mg 2 or 3 times daily orally one hour before meals.

Phenmetrazine hydrochloride

Tablets containing 25 mg and timed-release tablets containing 50 mg and 75 mg.

Recommended doses

Anorexic: Adults only: 25 mg 1 to 3 times a day, adminstered orally one hour before meals or 50 mg or 75 mg a day as a single prolonged-action tablet taken 1 hour before breakfast.

Benzphetamine hydrochloride

Tablets containing 25 mg and 50 mg.

Recommended doses

Anorexic: Adults only: from 25 to 50 mg 1 to 3 times daily. It is recommended to start with 25 mg and increase gradually as necessary.

Pipradrol hydrochloride (6, 7)

Tablets containing 1 and 2.5 mg.

Recommended doses

The usual starting dose for adults with depressive states is 2.5 mg 3 times daily, given orally. Elderly patients may receive 1 mg, once or twice daily.

Fentanyl citrate

Injection containing 0.05 mg of fentanyl base per ml, in 2 ml ampules.

Recommended doses

The manufacturer's recommended dose of fentanyl citrate for pre-operative medication is 0.05 to 0.1 mg (expressed as fentanyl base), administrated intramuscularly. The same dose is to be used post-operatively every 2 to 3 hours as required. By intravenous injection the equivalent of 100 to 600 micrograms of fentanyl (9).

Pethidine hydrochloride (4, 6, 7)

1) Meperidine hydrochloride injection (U.S.P.). A sterile solution of pethidine hydrochloride in water for injections pH 3.5 to 6.

2) Meperidine hydrochloride syrup (U.S.P.). A syrup containing pethidine hydrochloride pH 3.5 to 3.9. Store in airtight containers. Protect from light.

3) Meperidine hydrochloride tablets (U.S.P.). Tablets containing pethidine hydrochloride. Protect from light.

4) Pethidine injection (B.P.). Pethidine hydrochloride injection. A sterile solution of pethidine hydrochloride in water for injections. Sterilised by autoclaving.

5) Pethidine tablets (B.P.). Pethidine hydrochloride tablets. Tablets of 25 mg and 50 mg.

Recommended doses

a) Injection by subcutaneous or intramuscular, 25 to 100 mg; by intravenous, 25 to 50 mg.

b) Tablets: 50 to 100 mg.

Piritramide (7)

Available as 2 ml ampoules of an injection containing 10 mg per ml.

Recommended doses

20 mg intramuscularly appeared to be equal in analgesic effect to morphine. 15 mg intramuscularly.

Methadone hydrochloride (7)

1) Methadone hydrochloride injection (U.S.P.). A sterile solution of methadone hydrochloride 9.5 to 10.5 mg per ml in water for injections pH 3 to 6.5. Protect from light.

2) Methadone injection (B.P.). Amidone hydrochloride injection. A sterile solution of methadone hydro-

chloride injection. A sterile solution of methadone hydrochloride in water for injections. It is distributed in ampoules and sterilised by autoclaving 5 mg/ml and 10 mg/ml.

3) Methadone hydrochloride oral solution (U.S.P.). This is methadone hydrochloride 9 to 11 mg in each meal with a suitable preservative, it may contain suitable colouring and flavouring agents and surfactants pH 3 to 6. To be diluted with water before being taken. Store at 15° to 30° in airtight containers. Protect from light. Tablets of 5mg methadone hydrochloride.

Recommended doses

a) Injection by subcutaneous means, 5 to 10 mg.

b) Tablets: 5 to 10 mg

Normethadone hydrochloride

(same as Methadone hydrochloride)

Dipipanone hydrochloride (6)

a) Dipipanone injection B.P. A sterile solution of dipipanone hydrochloride in water for injection. The acidity is adjusted to pH 4.5 by the addition of hydrochloric acid. Sterilized by autoclaving. Protect from light.

b) Diconal tablets: Scored tablets each containing 10mg dipipanone hydrochloride and 30mg of cyclizine

hydrochloride.

Recommended doses

1) Tablets. Oral: One Diconal Tablet repeated every 6 hours, if necessary. If this dosage is not enough, it may be increased by increments of half a tablet.

2) Subcutaneous or Intramuscular injections of 12.5 mg to 25 mg of bipipanone hydrochloride repeated every 6 hours if necessary.

Norpipanone hydrochloride

Same as Dipipanone

1.5 METABOLISM, EXCRETION AND TOXICITY

It is now possible to suggest potential metabolites of a drug from its chemical structure. The metabolism of compounds related to amphetamine has been extensively examined because of experimental and clinical interests in the compounds (13, 14) and the possible involvement of metabolites in their pharmacology. The metabolic pathways for these compounds are of more general interest because amphetamine has structural elements common to many other classes of drugs. Metabolism of narcotic analgesics have also been investigated, but it is more difficult to have a general metabolic pathway for them as in amphetamine because most of them are not

structurally related and as such are treated on an individual basis.

Examining the structure of amphetamines suggests six main routes of metabolism (15, 16) while, amphetamine - a metabolite of methylamphetamine, suggests four routes, these are summarised in Fig. 1.1.

1. Aliphatic hydroxylation of the three carbon atoms of the side chain is pharmacologically important, although quantitatively minor, since its products are ephedrines. Unlike most other drug metabolism reactions, it is not carried out in the liver but in the noradrenergic nerves of the central and peripheral nervous systems, by the enzyme dopamine-β-hydroxylase. It is a stereoselective reaction restricted to the dextro-isomers which may account for the more pronounced pharmacological properties of the dextro compared with the laevo isomer.

2. Aromatic hydroxylation of the 2,3 or 4 positions of the benzene ring with the possibility of dihydroxylation . In fact it has only been observed in the para position and occurs extensively and consistently in the rat.

3. N-dealkylation of compounds bearing a substituent on the amino nitrogen. This is of the greatest quantitative significance and involves an oxidative process requiring molecular oxygen and NADPH. It occurs in the microsomal fractions of the liver cells

and gives rise to formaldehyde in equal molecular amounts to the primary amine formed.

4. Oxidative deamination to a ketone, which may then be reduced to an alcohol, and further oxidation leads to production of a benzoic acid.

5. N-Oxidation yields hydroxylamines, oximes and nitroalkanes.

Both oxidative deamination and N-oxidation occurs in microsomal fraction of the liver cells.

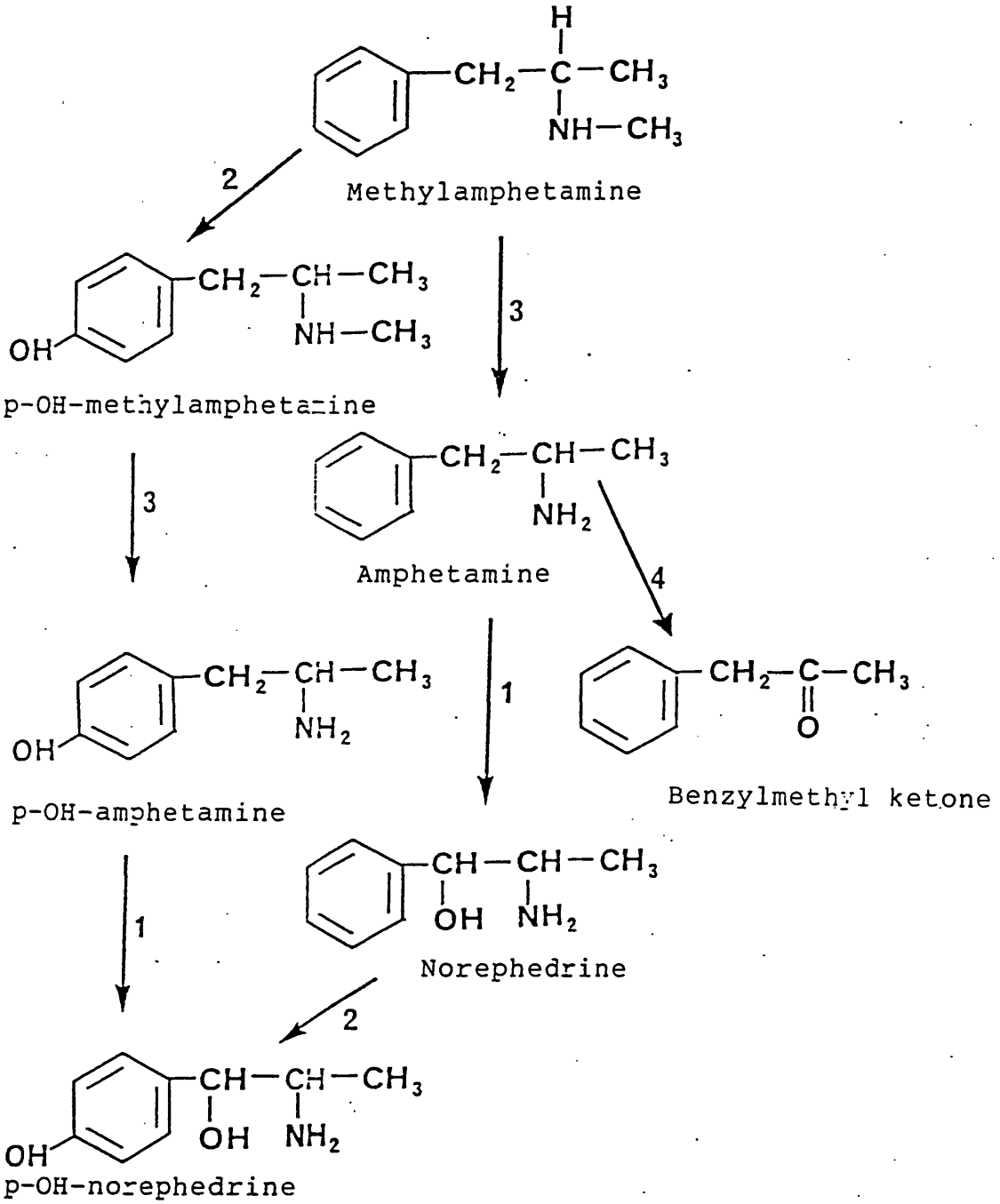
6. Conjugation of the amino nitrogen - the hydroxylated metabolites are generally excreted partly free and partly conjugated with either glucuronic or sulphuric acid depending on the species. In man, p-hydroxyamphetamine is excreted as its sulphate conjugate.

Methylamphetamine

It is readily absorbed after oral administration. About 70% of a dose is excreted in the urine in 24 hours. Under normal conditions up to 43% of a dose is excreted as unchanged drug, up to 15% as 4-hydroxymethylamphetamine, and about 5% as amphetamine which is the major active metabolite.

Excretion of unchanged drug is dependent on the urinary pH - the percentage is increased in acidic urine and greatly reduced (to about 2% of a dose) if the urine is alkaline.

Fig. 1.1. Metabolic pathways of methylamphetamine.



1. β-Hydroxylation
2. Aromatic hydroxylation
3. N-dealkylation
4. Oxidative deamination

Therapeutic Concentration

In plasma, usually in the range of 0.01 to 0.05 µg/ml.

Toxicity: The estimated minimum lethal dose is 1 gm

p-Hydroxyamphetamine

It is readily absorbed after oral administration. About 90% of a dose is excreted in the urine in 24 hours as free and conjugated hydroxyamphetamine, with about 4% of the dose as free and conjugated 4-hydroxy-norephedrine.

Toxicity: The estimated minimum lethal dose, intranasally in children up to 2 years of age is 200 mg and in adults about 2 gm.

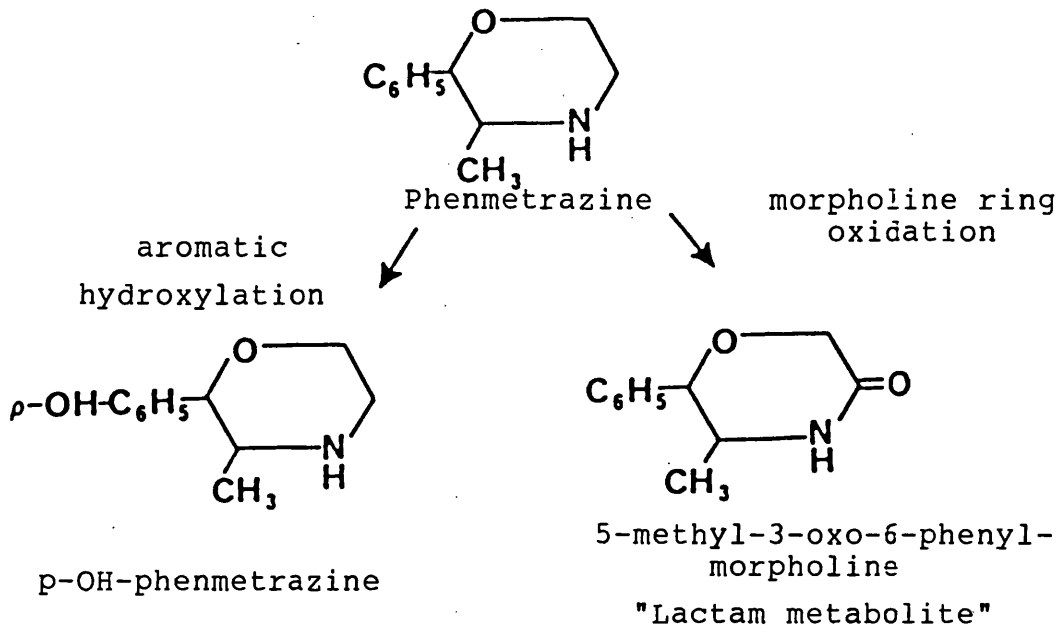
Phendimetrazine

It is readily absorbed after oral administration metabolised by N-demethylation to phenmetrazine, which is active, and by N-oxidation. About 5 to 30% of a dose is excreted in the urine unchanged in 24 hours together with up to 30% as phenmetrazine and 20% as the N-oxide. Plasma level is about 0.07 µg/ml in about one hour.

Half-Life: Plasma half-life is about 2 to 3 hours.

Phenmetrazine

Fig. 1.2 Metabolic pathways of phenmetrazine



Phenmetrazine is readily absorbed after oral administration. About 70% of a dose is excreted in the urine in 24 hours with about 19% as unchanged drug, 19% as the lactam (5-methyl-3-oxo-6-phenyl morpholine), 12% as free 4-Hydroxyphenmetrazine, 10% as conjugated 4-hydroxyphenmetrazine and about 5% as the N-oxide. Phenmetrazine is a metabolite of phendimetrazine. Plasma level concentration 0.16 µg/ml in about 2 hours.

Toxicity: Estimated minimum lethal dose for children up to 2 years of age is 200 mg.

Half-life: Plasma half-life is about 8 hours.

Chlorphentermine

It is absorbed after oral administration and metabolised mainly by N-oxidation and N-hydroxylation to nitro and hydyroxylamine derivatives. Excretion in urine is dependent upon urinary pH, a much greater proportion of unchanged drug is excreted when the urine is acidic than when it is alkaline. In normal urine, about 17% of a dose is excreted unchanged and about 50% as N-oxidised metabolites in 48 hours. If urine is maintained at an acidic pH, these proportions are approximately reversed, and about 70% of a dose will be excreted in 24 hours. In alkaline urine, less than 12% of a dose is excreted unchanged in 48 hours. Plasma level is about 0.32 µg/ml in 4 hours.

Half-Life: Plasma half-life is about 40 hours.

Phenethylamine

Phenethylamine is the putrefactive base obtained from phenylalanine by decarboxylation.

Mephentermine

Mephentermine is readily absorbed after oral or parenteral administration and rapidly metabolised by demethylation and hydroxylation. About 50 to 85% of a dose is excreted in the urine unchanged together with 15 to 20% as phentermine. Conjugated N-hydroxymephentermine has also been detected in the

urine in small quantities.

Toxicity: The estimated minimum lethal dose in children up to 2 years of age is 200 mg and in adults is 2 gm.

Half-Life: From urinary excretion data, the half-life of mephentermine is about 6 to 20 hours.

Benzphetamine

Benzphetamine is readily absorbed after oral administration and mainly excreted in the urine as amphetamine and methylamphetamine, very little is excreted as unchanged drug.

Fentanyl

This is rapidly metabolised in the liver. Two metabolites, norfentanyl and despropionylfentanyl, have been detected in plasma at concentrations similar to those of fentanyl. About 70% of a dose is excreted in the urine in 72 hours mostly as metabolites, with about 10 to 20% of a dose being excreted as unchanged drug in 48 hours. About 9% of a dose is eliminated in the faeces.

Toxicity: The estimated minimum lethal dose is 2 mg.

Half-Life: Plasma half-life is about 1 to 6 hours (dose dependent).

Pethidine

Pethidine is readily absorbed after oral administration and rapidly and extensively distributed throughout the tissues. The major metabolites are N-demethylated derivative norpethidine and the hydrolysis product, pethidinic acid and its conjugates. Excretion of pethidine and its metabolites is dependent on the urinary pH. In normal subjects about 70% of a dose is excreted in the urine in about 24 hours, up to 10% as unchanged drug, 20% as pethidinic acid, 16% as conjugated pethidinic acid and 8% as norpethidinic acid and 10% as conjugated norpethidinic acid.

Urinary excretion of pethidine and norpethidine may both be enhanced to about 30% of a dose if the urine is acid, in alkaline urine, less than 5% is excreted as pethidine and norpethidine. Usually, the plasma level concentration is about 0.2 to 0.8 µg/ml. Toxicity: The estimated minimum lethal dose is 1 gm. Half-Life: Plasma half-life is about 3 to 10 hours (mean 5) but it is increased in neonates and in renal impairment.

Methadone

Methadone is rapidly absorbed after oral administration and widely distributed in the tissues. The main metabolic reaction is N-demethylation resulting in a substance which spontaneously cyclises

to form the major metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3- diphenyl-1-pyrroline (EMDP), neither of which are active. In subjects on methadone maintenance, about 20 to 60% of a dose is excreted in the urine in 24 hours with up to about 33% of the dose as unchanged drug and up to about 43% as EDDP. EMDP is about 5 to 10% of the dose. Urinary excretion of unchanged drug is pH-dependent, it is increased in acid urine.

Plasma level concentration is about 0.05 to 1.0 µg/ml.

Toxicity: The estimated minimum lethal dose is 50mg.

Dipipanone

Dipipanone is absorbed after oral administration with rapid onset of action. It is excreted in the urine and faeces. Blood level concentration is about 1.6 µg/ml.

Toxicity: The estimated minimum lethal dose is 0.1 gm.

1.6. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1.6.1. History

Many forms of chromatographic methods are in use extensively as separative and analytical techniques. Gas chromatography seems to have dominated the scene since its introduction by Martin and James (17). Liquid chromatography has been developed into thin-layer,

ion-exchange, exclusion (gel filtration and gel permeation) and paper chromatography mode.Even though Giddings (18) in the mid 1960's had already shown advantages of liquid chromatography over gas chromatography, liquid chromatography on a comparable basis to gas chromatography is usually attributed to the work of Huber and Hulsman (19).

The success of gas-liquid chromatography and the need to develop silica packing materials caused some delay for the development of HPLC but since the developments of pellicular packings (20-22), porous silica and alumina microparticles of 5 μm, 10 μm and recently 3 μm (23, 24) and chemically bonded phases (25), the development of HPLC has progressed so rapidly that it is now the most extensively used chromatographic technique for separation, detection and quantification.

All these qualities are some of the main reasons why this technique has been used for the work carried out in this thesis.

1.6.2. Definitions

The following are some of the expressions that have been used in this thesis:

a. Band Broadening (Column Efficiency)

As a solute band progresses through a column, the

band width (w) increases and the solute is diluted by the mobile phase. This band width is normally used to calculate the theoretical plate number (N) of the column.

$$N = 16 \left(\frac{tr}{W}\right)^2 = 5.54 \left(\frac{tr}{W_{\frac{1}{2}}}\right)^2 \qquad (1.1)$$

where tr, W and $W_{\frac{1}{2}}$ are the retention time, width of the base and width at half the height of the peak respectively. The value of N is expected to be almost constant for different bands in a chromatogram, for a given set of operating conditions and is therefore a measure of the column efficiency (26).
This parameter was calculated for all the columns packed and used in this study.

In comparing column efficiencies a more useful parameter is the height equivalent to a theoretical plate, HETP or plate value H (which is a quantitative value for band spreading). The value N is proportional to column length L where:

$$N = \frac{L}{H} \qquad (1.2)$$

This value depends on the material and column design as well as the operating conditions. A very useful guide to column efficiency is the reduced plate height h given by

$$h = \frac{H}{dp} \qquad (1.3)$$

where dp is the diameter of the stationary phase particles.

b. Retention

Capacity ratio, k' is the term used to describe solute retention in column liquid chromatography. Retention and subsequently the separation of components in a mixture depends on the relative distribution of each component between the two chromatographic phases.

$$k' = \frac{C_s.V_s}{C_m.V_m} = K_D \quad \frac{V_s}{V_m} \qquad (1.4)$$

where C and V are the solute concentrations in and volumes of mobile phase (m) and the stationary phase (s) respectively. K_D is the distribution ratio of the solute between the two phases.
The capacity ratio may also be written as

$$k' = \frac{t_r - t_o}{t_o} \qquad (1.5)$$

where t_r and t_o are the elution times of the retained and unretained peaks respectively.

C. Resolution and Selectivity

The resolution, Rs of two adjacent solute bands is defined as being equal to the distance between the two band centres, divided by the average band width

$$R_s = \frac{t_2 - t_1}{\frac{1}{2}(W_2+W_1)} \qquad (1.6)$$

where t_1 and t_2 are the elution times of solutes 1 and 2 and W_1, W_2 are the respective widths at the base of their peaks. The above may also be expressed as

$$R_s = \frac{1}{4}\sqrt{N}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k'_2}{1+k'_2}\right) \qquad (1.7)$$

where k'_2 is the capacity ratio of the second peak, and α is the selectivity factor which is given by

$$\alpha = \frac{k'_2}{k'_1} \qquad (1.8)$$

d. Functional Group Contribution Value

Due to the importance of selectivity, Huber (76) suggested that there is a need to develop a reliable selectivity index, based on structural differences. This is then represented by

$$\tau = \log \alpha = \log \frac{k'_2}{k'_1} \qquad (1.9)$$

where k'_1 and k'_2 are capacity ratios of solutes 1 and 2 respectively which differ only by a functional group, where solute 1 is taken as the parent compound.

A functional group may contribute positively or negatively to retention, therefore τ may be positive or negative.

1.6.3 Stationary phases

Generally, liquid chromatography phase systems are classified as straight phase (or normal phase) and reversed phase depending on the nature of the stationary and mobile phases.

In straight phase systems, the stationary phase is more polar than the mobile phase, which is principally a mixture of organic solvents. In reversed phase systems, the stationary phase is non-polar whilst the mobile phase is polar.

In HPLC the stationary phase may be a porous solid like silica (24), carbon (28), polymeric resin (29,30) or most commonly a liquid chemically bonded to a porous silica material (31.32).

1.6.4 Retention Mechanisms

The different mechanisms by which separations may be achieved in liquid chromatography gives it major advantages over other separation techniques.

a) Liquid-Liquid (Partition) Chromatography

Liquid-Liquid or partition chromatography was developed by Martin and Synge. This method involves the use of a liquid stationary phase, which is obtained by filling the internal pores of a finely divided inert support. The sample to be analysed is dissolved in the mobile phase and its components are partitioned between the stationary phase and mobile phase according to their partition coefficients. Because of the variety of stationary phases available, this method is a versatile mode of liquid chromatography and a wide variety of compounds, both polar and non-polar can be separated.

b) Liquid-Solid (Adsorption) Chromatography

The original form of liquid chromatography was liquid-solid chromatography as developed by Tswett, using 'classical' column chromatography. Separation is by a liquid mobile phase and a solid stationary phase which reversibly adsorbs the solute molecules. The stationary phase may be either polar (e.g. silica gel, alumina) while the mobile phase would be relatively non-polar e.g. hexane or chloroform or it could be non-polar (e.g. Polymer beads) while a polar mobile would be used (e.g. water or ethanol). This latter method is known as reverse phase adsorption (75). The retention of samples in liquid-solid chromatography is

more predictable than in liquid-liquid chromatography since the elution order follows the polarity of the solutes. This is most successfully applied to non-ionic samples of intermediate molecular weight (200-2000) which are soluble in organic solvents. Ionic compounds usually give rise to 'tailing' peaks, because of the high surface energies of the adsorbents, unless the surface is deactivated in some way.

c. Bonded Phase Chromatography

This category has the most widely used materials in HPLC. These materials have hydrocarbon chains which are usually linear and bonded to the silica surface. The octadecysilane (ODS) derivative is the most cited material in HPLC literature and it has remarkable solute selectivity. Percentage of carbon loadings vary from 5 to 22%, the extent of silanol capping, the surface area, pore diameter and particle shape of the silica to which it is bonded all also vary. The mobile phases used are aqueous containing an organic modifier such as methanol or acetonitrile in order to reduce solute retention, and buffered at a pH that enhances selectivity between similar compounds. The retention mechanism is complex and as such not fully understood.

Horvath and Melander (33) proposed that solutes having some hydrophobicity are squeezed out of the aqueous mobile phase and into the hydrocarbon brush

because solute interactions with water are weaker than interactions between water molecules - this was called the Hydrophobic Effect.

This effect is increased with an increase in carbon loading and the carbon chain length. It has been observed that both increase in hydrophobic area of the solute and increase in mobile phase surface tension, cause increase in solute retention.

The control of pH is essential for ionisable solutes because solute-solvent interaction due to polar functions reduce retention. It has been shown by Scott and Kucera (34) that when an organic modifier is used to wet the surface of the bonded phase, then a monolayer of organic modifier is adsorbed at the hydrocarbon surface. Furthermore, they demonstrated that this layer is not displaced by solute and so must take part in the retention mechanism.

d) Ion-Exchange Chromatography

Ion Exchange chromatography which has been in use as a separative technique for some years now is a form of adsorption chromatography. The mode involves the substitution of one ionic species for another. The stationary phase consists of a rigid matrix, the surface of which carries a net positive charge to give an ion-exchange site (A^+) If a mobile phase containing anions is used, the exchange site will attract and hold

a negative counter-ion (B^-). Sample anions (D^-) may then exchange with the counter-ions (B^-). The process can be represented in terms of an equilibrium:

$$A^+B^- + D^- \rightleftharpoons A^+D^- + B^- \qquad (1.10)$$

This process is known as anion exchange. Bonded quaternary ammonium compounds give strong cationic groups suitable for exchanging acidic solutes, and similarly bonded alkyl sulphonic acid may be used to exchange cations.

Separation of samples is based on the strength of the interactions between the sample ions and the exchange site. Ions that interact weakly with the exchange site will be poorly retained and will have small k' values, while ions that have strong interactions will be strongly retained and will have high k' values.

e. Ion-Pair Chromatography

Ion-pair chromatography is a special form of liquid-liquid chromatography used for the separation of ionic or ionizable compounds (35, 36). In its most usual form, it is used in reversed phase mode with a hydrocarbon bonded stationary phase. Ion-pair extraction is a technique with which ionized compounds can be made to favour the organic phase during an extraction by using suitable counter-ions to form ion pairs according to the equation:

$$X^+_{aq} + Y^-_{aq} \rightleftharpoons XY_{aq} \rightleftharpoons XY_{org} \qquad (1.11)$$

where Y^- is the pairing-ion and the mobile and stationary phases are represented by subscripts aq and org respectively.

An extraction equilibrium constant, Ex,y may be defined as

$$E_{x,y} = \frac{[XY]_{org}}{[X^+]_{aq}[Y^-]_{aq}} \qquad (1.12)$$

The pairing-ion, Y^- is usually present in relatively high concentrations in the aqueous phase and is associated with its counter-ion. The solute X^+_{aq} is present in small concentrations. This demonstrates that retention of solute is increased as pairing-ion concentration is increased.

SECTION 2

EXPERIMENTAL

SECTION 2 EXPERIMENTAL

2.1 MATERIALS

2.1.1 Solvents

Methanol, acetonitrile, tetrahydrofuran, hexane, chloroform, propan-2-ol were of HPLC grade and were used without further purification. Pentan-1-ol was of Laboratory Reagent grade, and all the above solvents were supplied by Fisons Ltd., Loughborough, U.K.. Octan-1-ol was of Laboratory Reagent grade and supplied by Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. The water used for the preparation of aqueous mobile phases was double distilled from an all glass still and deionised by passage through an Elgastat deioniser, (Elga Products, Buckingham, U.K.). Deionised water was also obtained from Milli-Ro and Milli-Q water purification systems.

2.1.2 Acids, Bases, Buffer salts, reagents and ion-pair reagents.

Table 2.1.

COMPOUND	SUPPLIER
Acetic Acid	Fisons, Loughborough
Ammonia Solution	"
Disodium hydrogen orthophosphate	"
Orthophosphoric acid	"
Potassium chloride	"
Potassium dihydrogen orthophosphate	"
Potassium nitrate	"
Sodium hydroxide	"
Ammonium dihydrogen orthophosphate	BDH, Poole
Hydrochloric acid	May & Baker, Dagenham
Sodium dodecylsulphate	Cambrian Chemicals, Croydon
Sodium naphthalene-2-sulphonate	Eastman Chemical, Kodak Ltd., London

2.1.3 Solutes

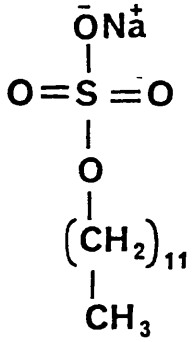
Table 2.2

COMPOUND	SOURCE
p-Hydroxynorephedrine	SKF
p-Hydroxyamphetamine hydrobromide	SKF
p-Hydroxy-N-methylamphetamine hydrochloride	SKF
Phenylethylamine	Aldrich
Dexamphetamine sulphate	C.R.E.
Chlorphentermine hydrochloride	C.R.E.
Methylamphetamine sulphate	C.R.E.
Mephentermine sulphate	C.R.E.
Phenmetrazine hydrochloride	C.R.E.
Phendimetrazine	C.R.E.
Benzphetamine hydrochloride	C.R.E.
Pipradrol hydrochloride	C.R.R.
Pethidine hydrochloride	May & Baker Ltd., C.R.E.
Piritramide	Janssen Pharmaceutical Ltd., C.R.E.
Fentanyl citrate	Janssen Pharmaceutical Ltd., C.R.E.
Normethadone hydrochloride	C.R.E.
Methadone hydrochloride	Wellcome, C.R.E.
Norpipanone hydrochloride	Wellcome, C.R.E.
Dipipanone hydrochloride	Wellcome, C.R.E.

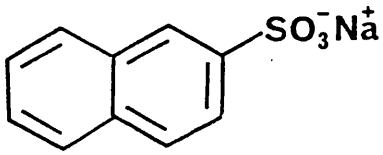
C.R.E. - Central Research Establishment
Forensic Science Service, Home Office,
Aldermaston

Fig. 2.1. Chemical Formulae

A. Pairing Ions

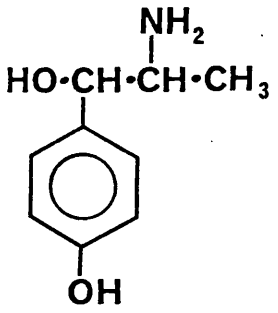


Sodium dodecyl sulphate (SDDS)

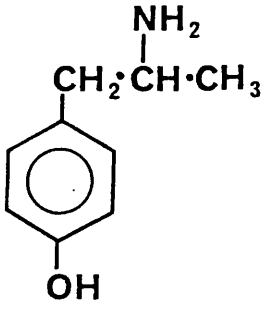


Naphthalene-2-Sulphonate Sodium Salt (NS)

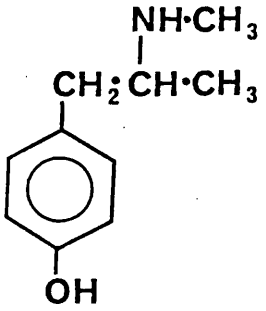
B. Solutes (1)



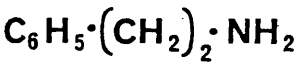
p-Hydroxynorephedrine



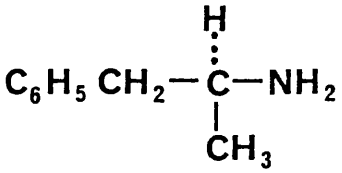
p-Hydroxyamphetamine



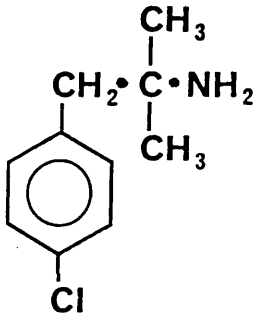
p-Hydroxy-N-Methylamphetamine



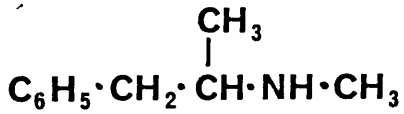
Phenylethylamine



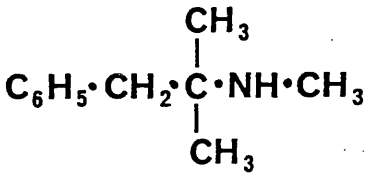
Dexamphetamine



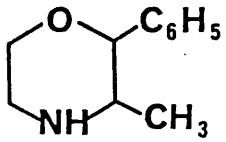
Chlorphentermine



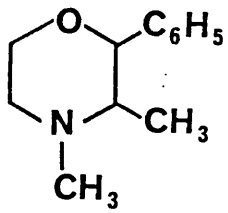
Methylamphetamine



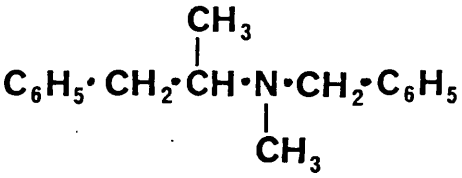
Mephentermine



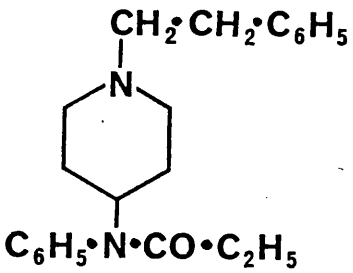
Phenmetrazine



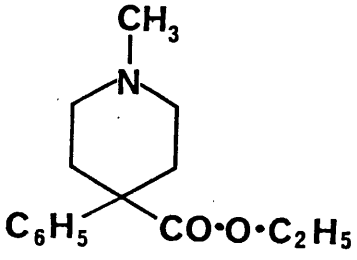
Phendimetrazine



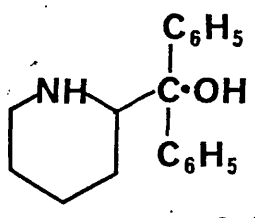
Benzphetamine



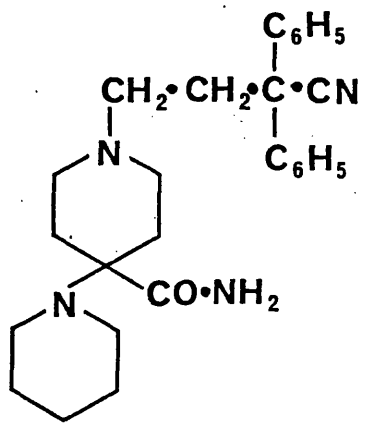
Fentanyl



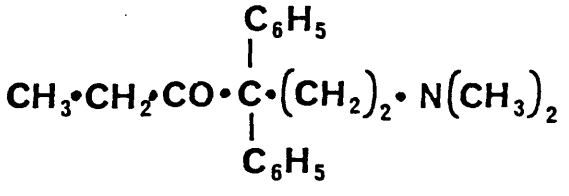
Pethidine



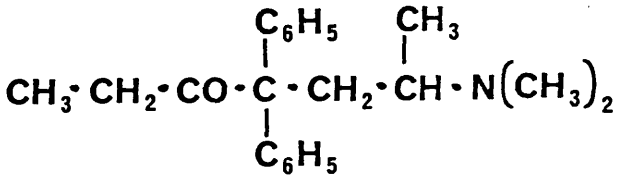
Pipradrol



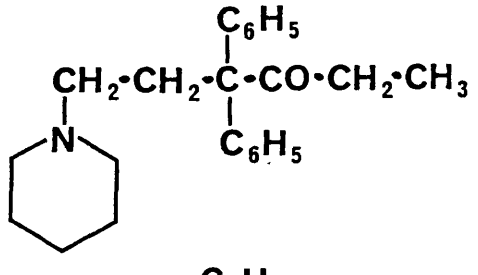
Piritramide



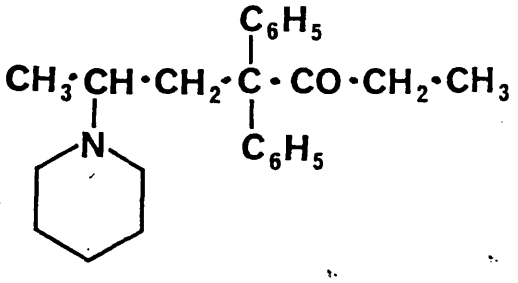
Normethadone



Methadone



Norpipanone



Dipipanone

2.1.4 HPLC packing materials

Table 2.3. Packing materials used

PACKING MATERIAL	MANUFACTURER
Spherisorb 5 CN	Phase Sep.
Partisil SCX	Whatman Labsales
Nucleosil SA	Macherey-Nagel
PRP-1	Hamilton
Hypersil SAS	Shandon
Hypersil phenyl	Shandon
Hypersil ODS	Shandon

Table 2.4. Summary of columns employed in this study. Column diameters were 4.6 mm except for column E which was 2 mm.

Column Code	Packing Material	Length (mm)	Particle size (µm)
A	Spherisorb-CN	50	5
B	Partisil-SCX	150	10
C	Partisil-SCX	100	5
D	SAS-Hypersil	100	5
E	SAS-Hypersil	100	5
F	Nucleosil SA	100	5
G	Nucleosil SA	50	5
H	PRP-1	150	10
I	ODS-Hypersil	150	5
J	Hypersil-Phenyl	100	5

Table 2.5. Summary of some of the properties of the packing materials used.

Column Code	Functionality	% Carbon (w/w)	Surface Area (m^2/g)	Pore Size (nm)	Particle Shape	End Capping
A	Cyanopropyl-	3.5	200	8	Spherical	Not known
B + C	Aromatic benzenesulphonic acid		350	8.5	Irregular	Nil
D	Trimethyl-	2.6	200	12	Spherical	Not
E	Trimethyl-	2.6	200	12	Spherical	required
F + G	benzenesulphonic acid			10	Spherical	Nil
H	divinylbenzene		415	7.5	Spherical	Not required
I	Octadecylsilane-	10.0		12	Spherical	Full
J	Phenylpropyl-	5.0	220	8	Spherical	Not known

2.2 EQUIPMENT

2.2.1 HPLC Instrumentation

The HPLC instrumentation used was assembled from commercially available components. Fig. 2.3 shows a generalized scheme of the liquid chromatograph used. The pump used was either a Constametric III or a Milton Roy Minipump with pulse dampener (both from LDC, Stone). Sample injections were made by means of 50ul and 100µl syringes (Hamilton type 75N, Phase Separations, Queensferry)through a valve injector (Rheodyne 7125, Jones Chromatography, Llanbradach) having a 20µl loop volume.

The column eluent was monitored by UV-Spectrometry, using the following detectors:- Cecil CE212, Cecil 2102 variable wavelength spectrophotometer (Cecil Instruments, Cambridge, England) U.V-Spectrophotometer (Du Pont Instruments, Herts, England). A Perkin-Elmer 550-S UV-Vis spectrophotometer (Perkin-Elmer Ltd., Slough, England) was used for non-HPLC spectroscopic measurements.

The chromatography columns consisted of both 2mm and 4.6mm internal diameter (¼" external diameter) stainless steel tubes of various lengths (supplied by HETP, Macclesfield, U.K.). The column end fitting consisted of a low dead-volume swagelok connection with a 1/16" outlet which was connected to the detector by microbore (internal diameter 0.15 mm) Teflon tubing.

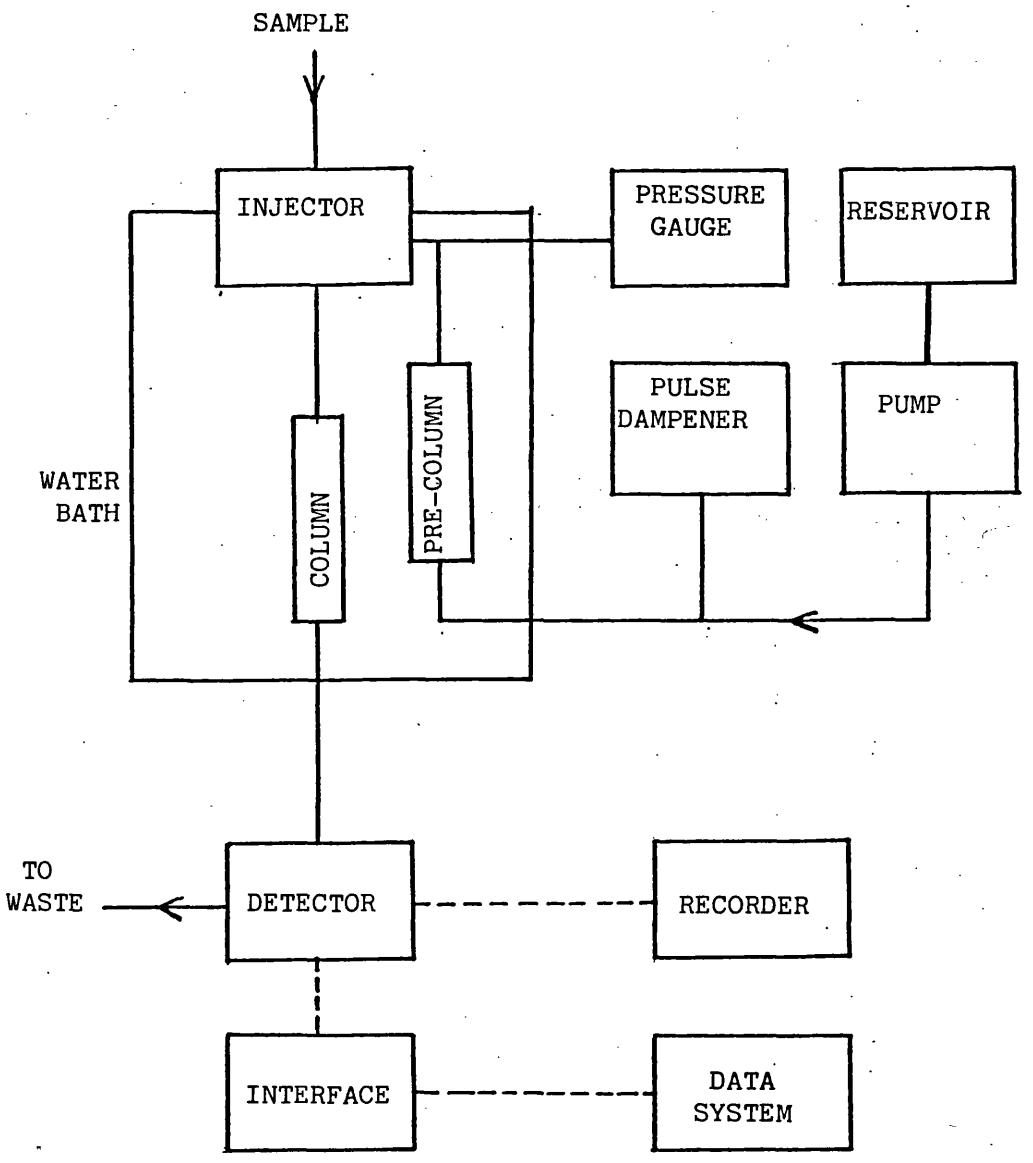


Fig. 2.3. Generalised Scheme of the Liquid Chromatograph.

Low dead-volume 1/16" stainless steel tubing (internal diameter 0.15 mm) was used to connect the injection valve to the top of the column. Other connections were made using stainless steel tubing and standard swagelok fittings (HETP). All connections were made as short as possible.

The temperatures of the columns, injector and mobile phase reservoir were controlled by immersion in a heated water bath (Gallenkamp type 400-010)wherever practical.

2.2.2. HPLC Column packing apparatus

The column packing apparatus (see Fig. 2.4) consisted of a stainless steel 'bomb' 75 ml in volume which served as the slurry reservoir. One end of the 'bomb' was connected to a pneumatic amplifier pump (SAT RIG column packer, Haskel Energy Systems Ltd.) and the other end was attached to a ¼" external diameter stainless steel guard column. Iso-propanol and hexane were used for column packing. Procedure is described in section 2.3.1.

2.2.3. Segmented flow system

A generalised scheme of the system is shown in Fig. 2.5.

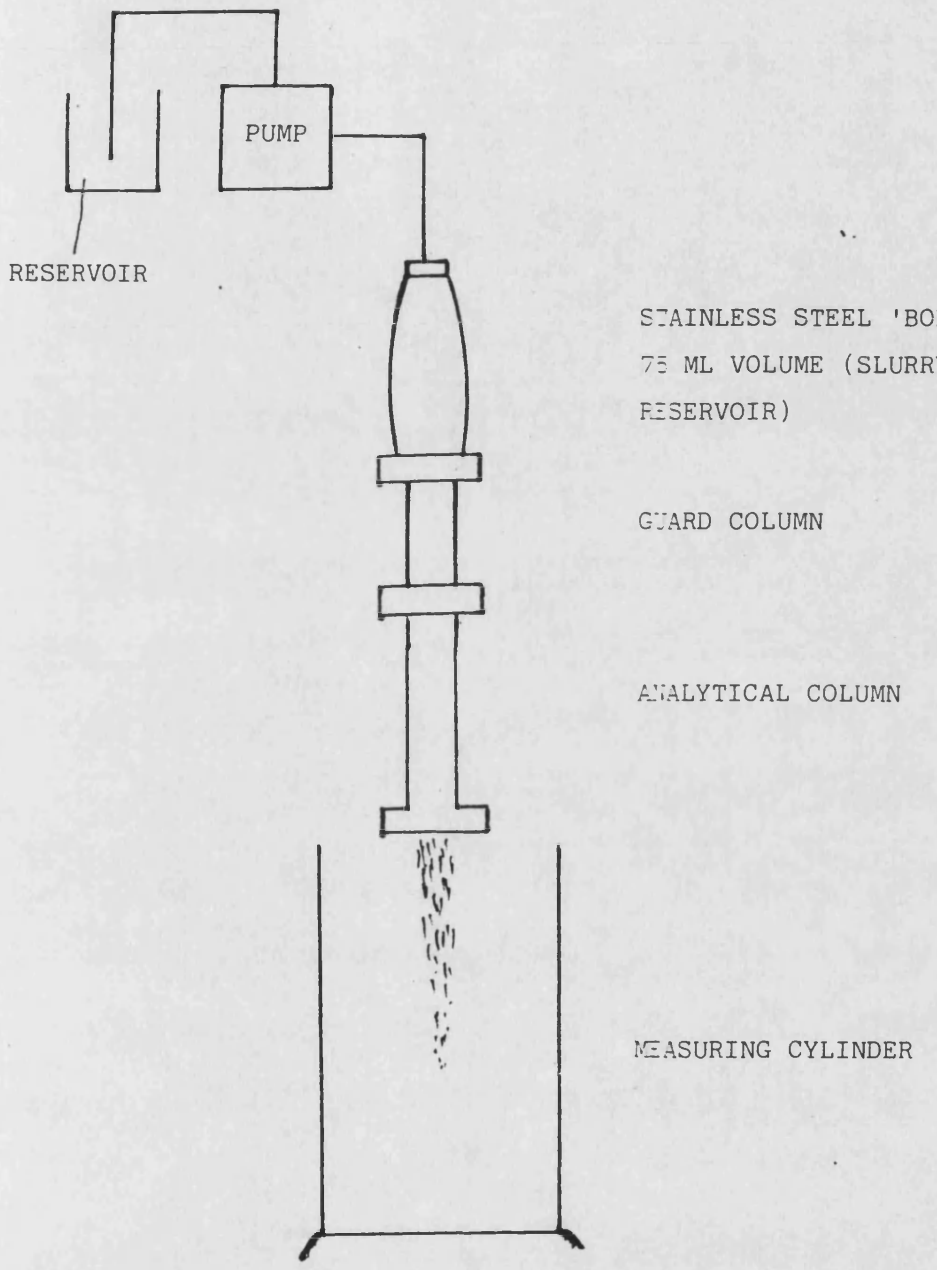


Fig. 2.4. Column Packing Apparatus

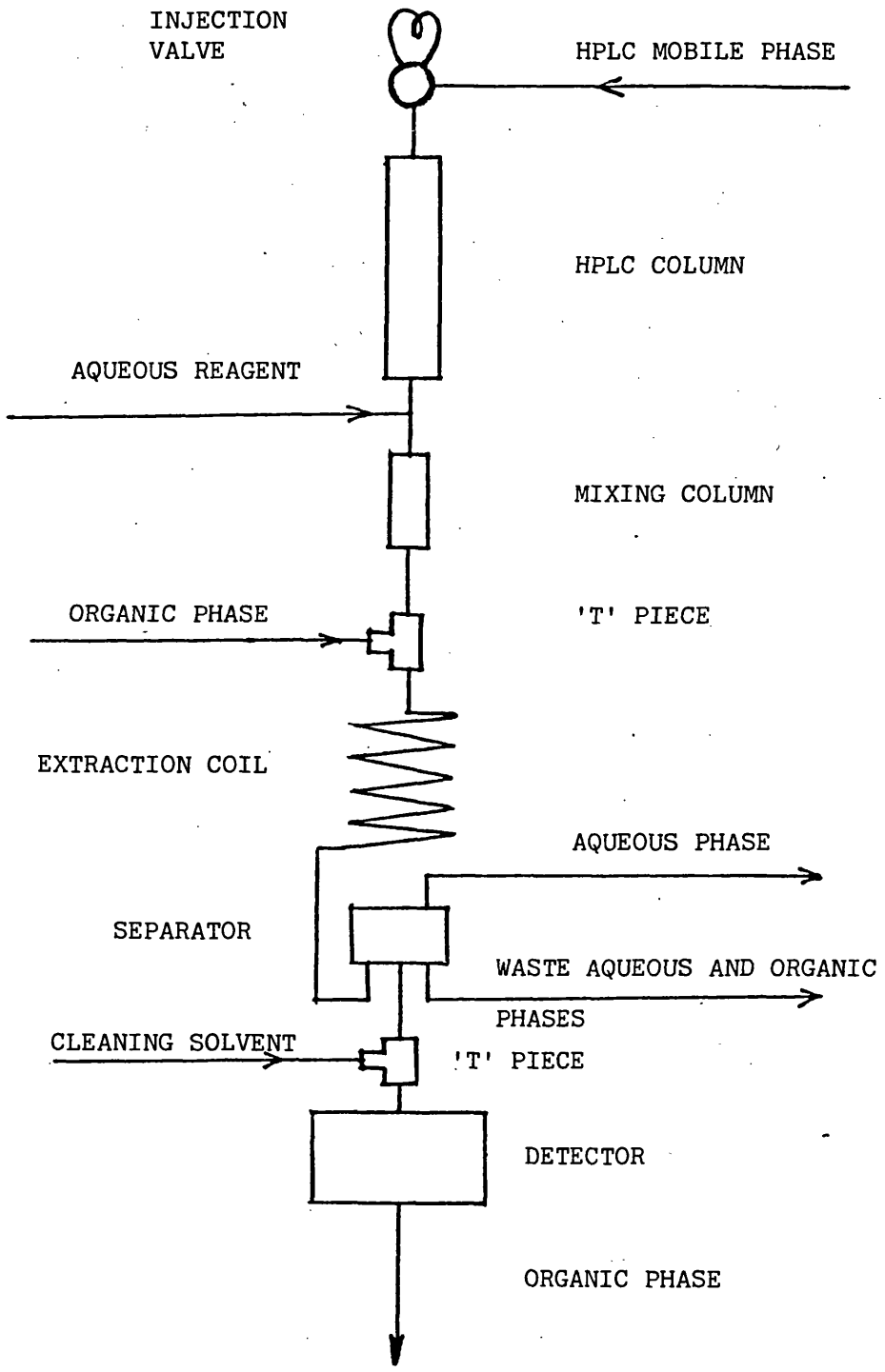


Fig. 2.5. HPLC with Post-Column Reactor

(a) Pumping devices

The organic phase was pumped into the system via a coiled ¼" stainless steel tube which was pressurised from a cylinder of nitrogen gas via control valves, and the flow rate of the organic solvent was then adjusted by changing the pressure of the nitrogen gas. This equipment was a gift from ICI Pharmaceuticals Ltd., Macclesfield, Cheshire.

A multi-channel peristaltic pump (Gilson Minipulse-2; Anachen, Luton, U.K.) was used to create suction for the aqueous organic and waste lines of the phase separator when necessary.

(b) Phase separator

Two types of phase separators of slightly different designs were used in this work. Phase separator I was made to a design described by Kinkel and Tomlinson (37), which was a modification of a design by Copsey (38). It consists of one surface of PTFE and another of glass both surfaces being channelled to make a separating chamber of aproximately 28 µl in volume. A cross- section is shown in Fig. 2.7. The two surfaces are enclosed in a cylindrical brass jacket made to hold the glass and PTFE blocks together tightly to prevent leakage at normal flow rates. The orientation of the separator is chosen so that the liquid phase with the highest density wets the

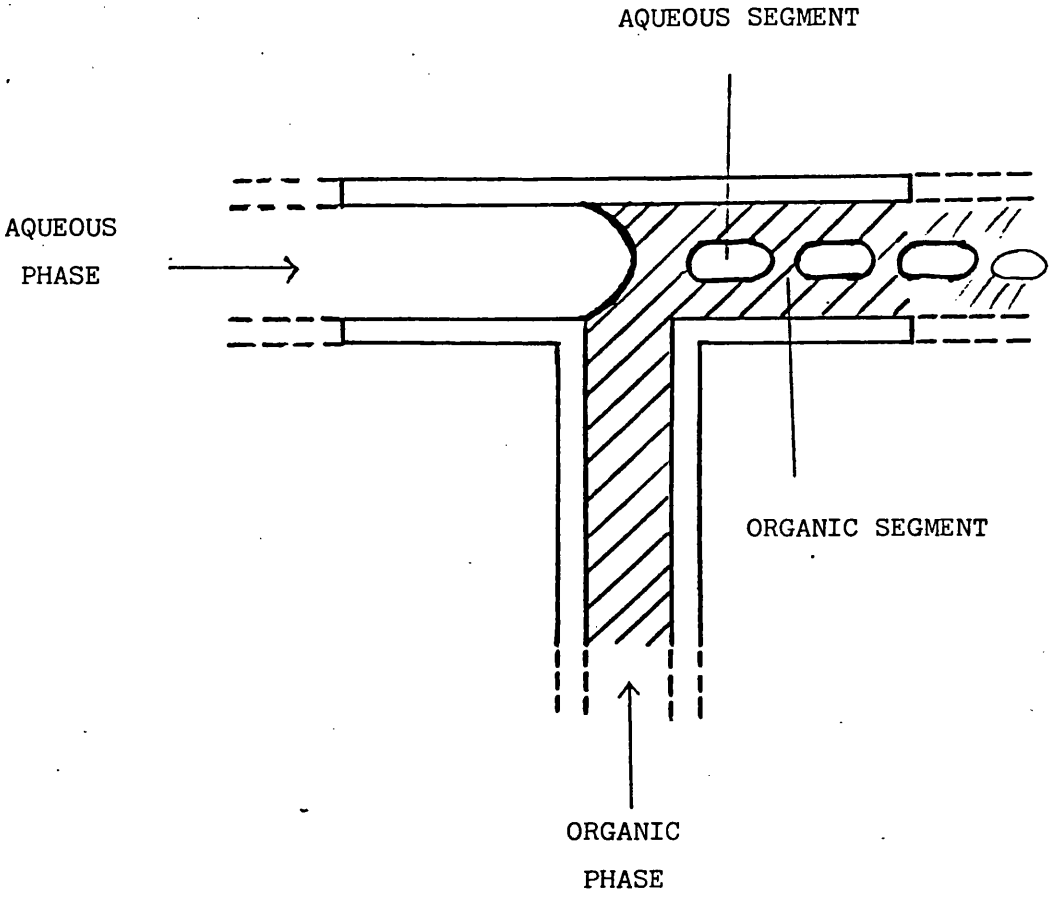


Fig. 2.6. 'T'-Piece segmentor and segments produced.

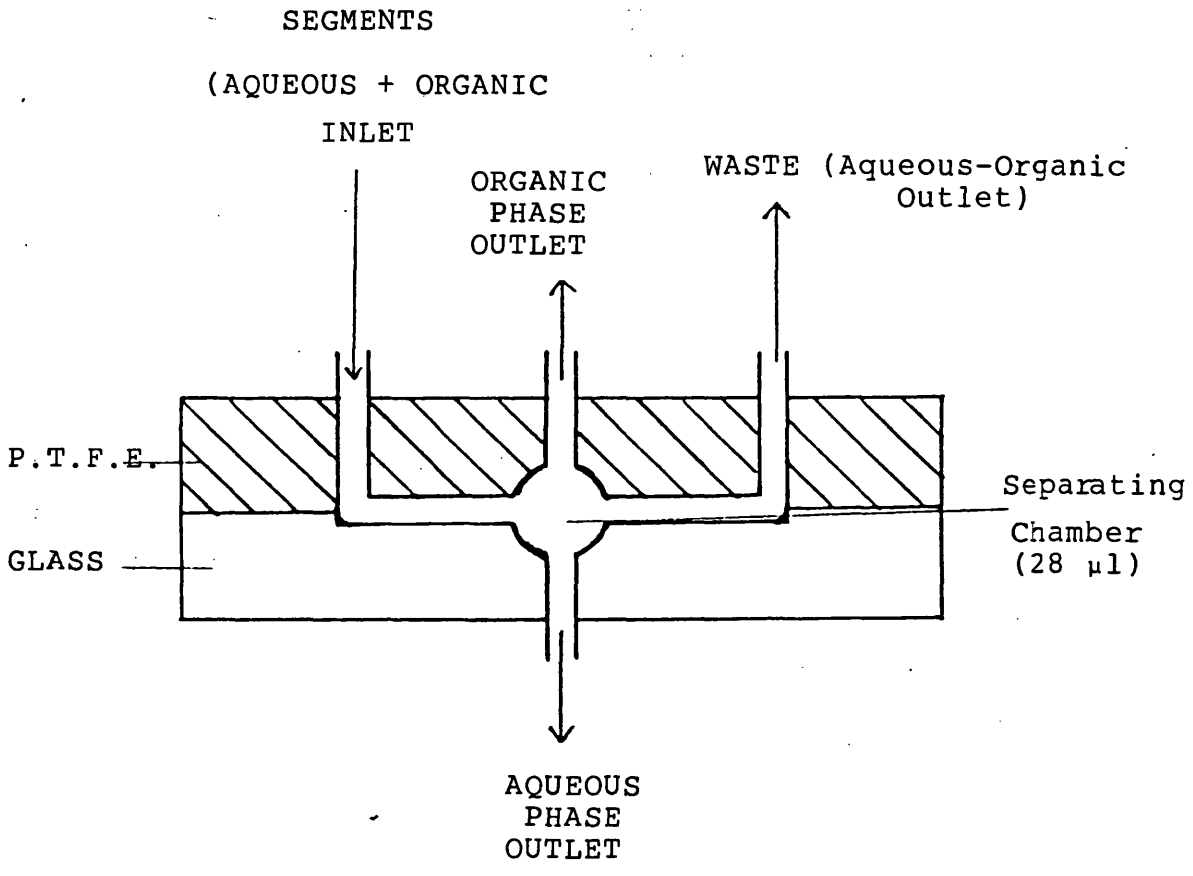


Figure 2.7. Cross-Section of Phase Separator I.

surface of the lower block (glass or PTFE).

Phase separator II was designed by T.M. Jefferies, (Bath University). It is similar to phase separator I but is larger and has extra channels for cleaning-up any droplets of aqueous phase from the organic phase and vice-versa. A cross-section is shown in Fig. 2.8.

2.2.4 Flow-Injection Analysis

The post-column reactor (Fig. 2.5) including the phase separator, may be used independently without an HPLC column as a flow-injection analysis system. Aqueous reagent is pumped directly through the injection valve to a mixing column.

To start up the system, organic phase only was supplied to ensure the absence of leaks and the absence of water droplets in the detector. Aqueous phase was then supplied, either by using the HPLC system or an independent pump or the peristaltic pump as required.

The system can be used for the determination of physico- chemical properties such as partition coefficient and pKa values. The transfer of a solute into an organic phase can be measured as previously described following an injection of the solute into the aqueous system (Pulse Input), or by pumping an aqueous solution of the solute into the organic phase (Step Input). It permits a sufficient volume of organic phase-containing solute - to be collected and studied separately by spectrophotometer.

Figure 2.8.

Configuration of phase separator for monitoring the organic phase.

Flowpath of organic phase:

1. Aqueous/organic segmented flow inlet
2. Separated organic phase plus some aqueous droplets
3. Re-entry into second separating channel
4. Exit of clean, organic phase to detector

Flowpath of aqueous phase:

1. Aqueous/organic segmented flow inlet
5. Exit of aqueous phase
6 Exit of any water droplets + some organic phase

The separator may also be configured to monitor the aqueous phase by connecting exit 5 to re-entry 7 and connection of exit 8 to the detector.

Exits 9 and 10 are for "waste" organic phase

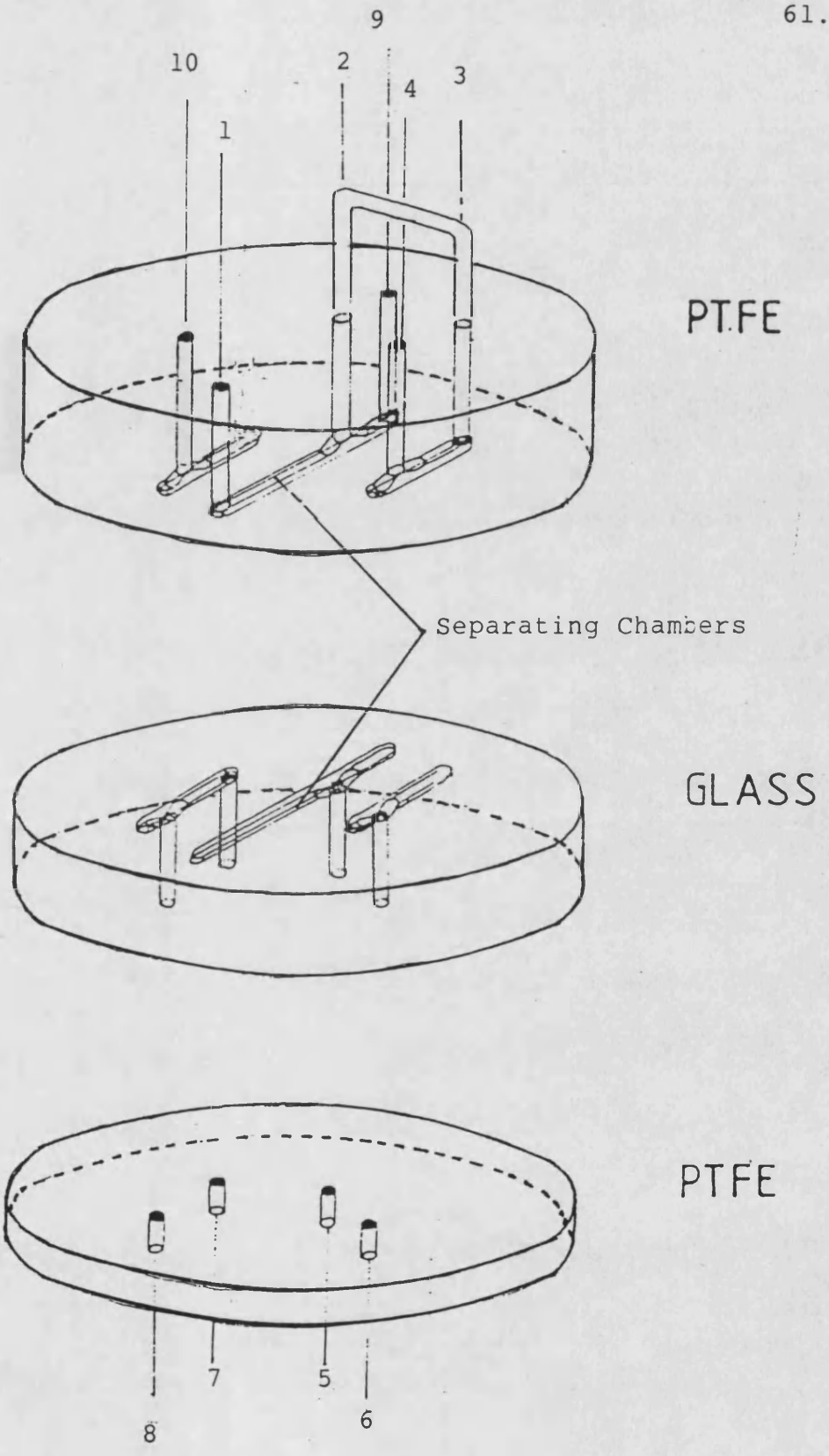


Figure 2.8. Three-dimensional diagram of Phase Separator II. Legend on opposite page.

2.3. GENERAL PROCEDURES

2.3.1. Column packing and preparation

Most of the columns used were packed, using the equipment described in Fig. 2.4, employing iso-propanol to make the slurry and hexane as the packing solvent (recommended by Shandon, Cheshire, U.K.). Column lengths were either purchased already treated with nitric acid, or if not, were soaked in 2N nitric acid for 1-2 hours and rinsed, and dried. After that, the bottom end of the column was fitted with mesh and ferrule while the top is fitted with only the ferrule. The 'bomb' (slurry reservoir) is then cleaned with methanol and dried with compressed (pressurised) air. To pack a column (100 mm x 4.6 mm i.d), 1.4 g of the packing material was weighed into a 50 ml beaker and dispersed in 25 ml of iso-propanol by immersion in an ultrasonic bath. The reservoir was filled with hexane. The head pressure of the nitrogen cylinder was turned up to 120 lb/in^2 and the pump was set to recycle, to completely fill the system with the packing solvent. With the bottom end of the column closed, it was filled with iso-propanol and then attached to the slurry reservoir.

The slurry was then added to the slurry reservoir to completely fill the reservoir. The slurry reservoir and column were then attached to the pump. The pump was then charged to a pressure of 7000 p.s.i., the

column end opened and the valve was then released. About 300 ml of hexane was pumped through the column at the set pressure and then reduced gradually to zero. The valve was then shut-off and the column allowed to stand for about 10 minutes. It was then removed and topped with excess material from the 'bomb' before a 2 μm stainless steel mesh frit was placed on top of the packing before swageing on the column end. The column was then washed with methanol and stored until required.

The mobile phase is usually recycled through the column for some hours before use and where an ion-pair reagent is present, then the pump is run overnight to stabilise the column.

2.3.2 Preparation of mobile phases

Aqueous mobile phases were prepared by pipetting the appropriate amounts of organic modifier, buffer and pairing-ion (when used) solutions into grade B volumetric flasks and adjusting to volume with double distilled, deionised water. The final volume adjustment was made after cooling the mobile phase to room temperature. The pH of the mobile phase was adjusted by dropwise addition of (85%) H_3PO_4 or ammonia solution (98%) and the pH was measured using a PTI-6 Universal Digital pH Meter. The pH of solutions prepared in this way are denoted in this thesis by pH*.

In other cases the pH of purely aqueous buffers were adjusted and then organic solvent added. Before the mobile phase was used, it was filtered through a filtering apparatus with a 0.45um pore membrane filter, and purged with helium for 10 minutes to remove dissolved gases in solution.

2.3.3 Preparation of Organic phase

The solvent used for the segmented flow experiments was a mixture of chloroform and pentan-1-ol (90 + 10 v/v). This mixture was equilibrated with the aqueous phase (aqueous reagent and mobile phase). Usually the organic phase was shaken for 15 minutes with aqueous phase in a separating flask, the organic layer removed, filtered and degassed before use.

2.4 EXTRACTION OF DRUGS FROM BIOLOGICAL FLUIDS

a) Protein precipitation

To plasma (0.5 ml) 5% trichloroacetic acid (2 ml) was added and shaken thoroughly. The precipitated plasma proteins were separated from the solution by centrifugation at 3000rpm for 15 minutes. The clear supernatant was removed for extraction using XAD-2 resin.

b) Extraction

Samples of supernatant, up to 10 ml, were made

alkaline with 5N-sodium hydroxide to pH 12.0 (pka+2) e.g. Chlorphentermine, Normethadone, Dexamphetamine. The solutions were then introduced onto 'columns' (5 ml graduated pipettes) each containing about 1.5 g of clean XAD-2 resin (Amberlite, B.D.H. Poole, U.K.). The resin had previously been washed with methanol, acetone and water. These columns produced flow rates of about 2.5 ml/min. The columns containing the adsorbed solutes were washed with 10ml of water and then eluted with 20 mls of chloroform: isopropanol (3:1). Ethanolic hydrochloric acid 6N (100µl) was added to the organic eluate which was then evaporated to dryness under vacuum using a rotary evaporator (Buchi Rotavapor-R). The residue was then dissolved in a known quantity of the H.P.L.C. mobile phase for direct injection or post column derivatisation.

2.5 ANALYTICAL PROCEDURES

Known quantities of compounds of interest were spiked into plasma and were assayed after the appropriate pretreatment by comparison with analytical standards of known concentration. The assays were performed using one of the compounds as internal standard in as much as the compounds did not interfere with each other (i.e. well separated). A single standard solution of the compounds was injected ten times to determine precision of the injection

procedure. Standard deviation of the peak heights, or peak height ratios were determined.

Peak height ratios of 5 to 8 standard solutions containing a range of solute concentrations were used to prepare calibration graphs. The maximum concentration ranges used were up to twice the expected concentration. Each solution was injected in triplicate and the data fitted to:-

$$y = mx + c \qquad (2.1)$$

where y is the peak height ratio, x is the concentration of solute and m and c are regression coefficients for slope and intercept respectively. The concentrations of the unknown samples analysed were determined by comparison of their response with the responses obtained from two or three analytical standards of known concentration whose linearity of response had previously been confirmed.

2.5.1 Post column derivatisation for Ultra violet detection

Ion-pair reagent solution of appropriate concentration was filtered and well degassed with helium before being pumped to the T-connection at the end of the H.P.L.C. column.

Both pH and flow rate of the reagent were adjusted such that the desired pH for ion-pairing with the compounds eluted from the H.P.L.C. column was achieved.

The derivatisation reagent and H.P.L.C. eluent were then passed into a mixing column (50 mm x 4.6 mm I.D) to assist rapid and uniform ion-pair formation to occur. The mixing column was cooled with ice when necessary to stabilise baseline noise in the detector, caused by chromatography carried out at temperatures above room temperature. This then passes on to mix with the immiscible organic solvent via a 'T'-piece (Fig. 2.6) which forms the segments which subsequently passes through the extraction coil (Fig. 2.5) and then the phase separator after which the phase of interest is monitored through the UV-detector.

2.6 DETERMINATION OF IONISATION CONSTANTS

The method used was that described by Albert and Serjeant (58). A solution was made of the amine compound to be measured at a concentration of 0.5 mmol in 50 ml, (i.e. 0.01 M solution). This volume was placed in a 100 ml beaker and titrated with 0.1 M sodium hydroxide, which was added in 0.5 ml portions and the pH values were recorded. A total of 5 ml base was added and calculations were made to correct for the changes in stoichiometric concentrations so as to give a maximum of nine values for the pKa. The pKa values of each compound were determined in different solvent compositions and temperatures and are listed in Table 4.2.

The pH values were measured using a thermometer electrode and a combined glass/calomel electrode connected to an EIL Model 23A direct Reading pH meter. Each determination was made in duplicate.

In order to gain an appreciation of the comparability of the results obtained using the above equipment and technique the pKa values of several substances were determined with published values as standards.

2.7 DETERMINATION OF PARTITION COEFFICIENTS

Several methods by different authors have been described for partition coefficient determinations, and the most extensively used method is by simply shaking a solute with two immiscible solvents and then analyzing the concentration in one or both phases. Partition coefficients in octanol-water systems are frequently used for characterization of hydrophobic properties of drugs. It is claimed that the 1-octanol-water system is a satisfactory model for the biological system, because the organic phase is not completely non-polar and contains a significant amount of water in a stable, hydrogen-bonded complex.

The method used here is that described by Leo et al (40). The two immiscible phases used for the experiments are:- Octan-1-ol and the appropriate buffer solution at chromatographic pH without the organic

modifier. The two phases were equilibrated with each other and filtered prior to dissolution of 10^{-4} - 10^{-2}M solute in the aqueous buffer phase. The organic phase and the aqueous phase containing the solute were shaken by simple inversion 100 times in five minutes in a 50ml centrifuge tube fitted with a glass stopper.

A calibration was prepared for each compound over the concentration range 10^{-4} - 10^{-2}M in aqueous phase. The absorbance of a known concentration of solute in aqueous phase was then determined before partition with octan-1-ol and then after partition and centrifugation; the quantity left in the aqueous phase was determined by relating the absorbance to its concentrations on the calibration plot. The partition coefficients is then calculated as follows:-

$$P = \frac{C_o}{C_w} \qquad (2.2)$$

where Co is the concentration of the solute in the organic phase, and Cw is the concentration of the solute in the aqueous phase.

SECTION 3

RESULTS AND DISCUSSION

THE EXAMINATION OF VARIOUS STATIONARY PHASES FOR THE ANALYSIS OF SOME DRUGS OF ABUSE

SECTION 3. RESULTS AND DISCUSSION

THE EXAMINATION OF VARIOUS STATIONARY PHASES FOR THE ANALYSIS OF SOME DRUGS OF ABUSE

3.1 Cyanopropyl-bonded silica

3.1.1 The Stationary Phase

The basis of all the bonded silica materials is the unbonded silica, which for Spherisorb materials is a totally porous, spherical material having a surface area of 220 m^2g^{-1} and a mean pore diameter of 8 nm (range 5.4 to 11 nm). It is available in three particle size ranges (3, 5 and 10 μm).

Cyanopropyl-bonded silicas are generally marketed as normal phase materials intended for the chromatography of polyfunctional aromatic compounds, using organic solvent mixtures such as hexane and propan-2-ol. Under these conditions, it is a weaker hydrogen-bonding adsorbent than underivatized silica and so produces narrower peaks for these compounds. For example:

Solute-Solvent	Solute-Cyano-silica
R - OH·····:NC.CH_3	R-OH·····:NC-$CH_2CH_2CH_2$-Si-

However, they have properties that make them suitable for rapid chromatography using reversed-phase conditions.

The percentage carbon loading is low, for example, for the Spherisorb range of -CN, -ODS1 and ODS2 derivatized silicas, the values are 3.5, 7.0 and 12.0% w/w respectively, whilst the -CN surface coverage is better (0.6 mM/g) compared to the ODS materials (0.3 and 0.5 mM/g respectively) which helps to minimise the effect of any underivatised silanol groups. Additionally the -CN group introduces polar properties (41) (log P = -1.07) onto the propyl chain (3 x $-CH_2-$, log P = 1.59) that should reduce solute retention when compared to a propyl chain plus a terminal $-CH_3$ group (log P = 0.70). It also permits solutes to interact by hydrogen-bonding and thus adds 'different' selectivity.

To further reduce solute retention a short column (50 mm x 4.6 mm i.d.) was packed and used at 30°C rather than at room temperature. This should then provide chromatographic conditions that may also be employed for post-column reactions designed to improve solute detectability. Preliminary investigation showed that the phase separator unit would be unable to clearly separate chloroform from an aqueous phase containing more than 25 percent v/v methanol or 25% v/v methanol-acetonitrile mixture. Another restriction in the choice of chromatographic conditions was the need to employ low concentrations of buffers since cations such as Na^+ and K^+ tended to facilitate the transfer of pairing-ions from an aqueous to an organic phase. Since

it was intended to employ the highly UV-absorbing compound naphthalene-2-sulphonic acid to transfer amines into the chloroform phase, the unwanted transfer of ion-pair reagent by buffer salts would generate a high UV-absorbing background signal. This would make the post-column system difficult to stabilise or operate at high sensitivity levels.

3.1.2 Investigation of chromatographic conditions using cyanopropyl-bonded silica

Chromatographic conditions were investigated to produce good resolution within as short a chromatographic run-time as possible. As a starting point, it was decided to employ a short column of 50 mm length with equal volumes of methanol and acetonitrile diluted with 0.05 M phosphate buffer.

3.1.2a Influence of Mobile Phase pH

In view of the importance of mobile phase pH, its influence on the retention of Group A solutes was studied using 0.05 M phosphate buffer-methanol-acetonitrile (90:5:5, v/v/v). Retentions of all solvents (Fig. 3.1) fell with pH as expected, but did not reach their minimum values over the pH range (5.4-7.2) of this buffer. It has been reported (42) that the pKa values of 30 basic drugs when measured in methanol-aqueous buffer (pH 4 or 7) were lower than

Figure 3.1 Influence of mobile phase pH on the retention of some Group A compounds. Conditions: 5 µm Spherisorb-CN column (50 x 4.6 mm i.d.) with 0.05 M phosphate buffer-methanol-acetonitrile (90:5:5, v/v/v) at 30°C. Injection volume 10 µl, flow rate 1 $ml.min^{-1}$, monitored at 205 nm with 0.04 a.u.f.s.

Codes and Literature pKa for Group A Compounds

No	Compound	pKa
1	p-Hydroxynorephedrine	-
2	p-Hydroxyamphetamine	9.3
3	p-Hydroxymethylamphetamine	-
4	2-Phenylethylamine	9.83
5	Dexamphetamine	9.90
6	Phenmetrazine	8.40
7	Methylamphetamine	10.10
8	Mephentermine	10.40
9	Phendimetrazine	7.60
10	Chlorphentermine	9.60

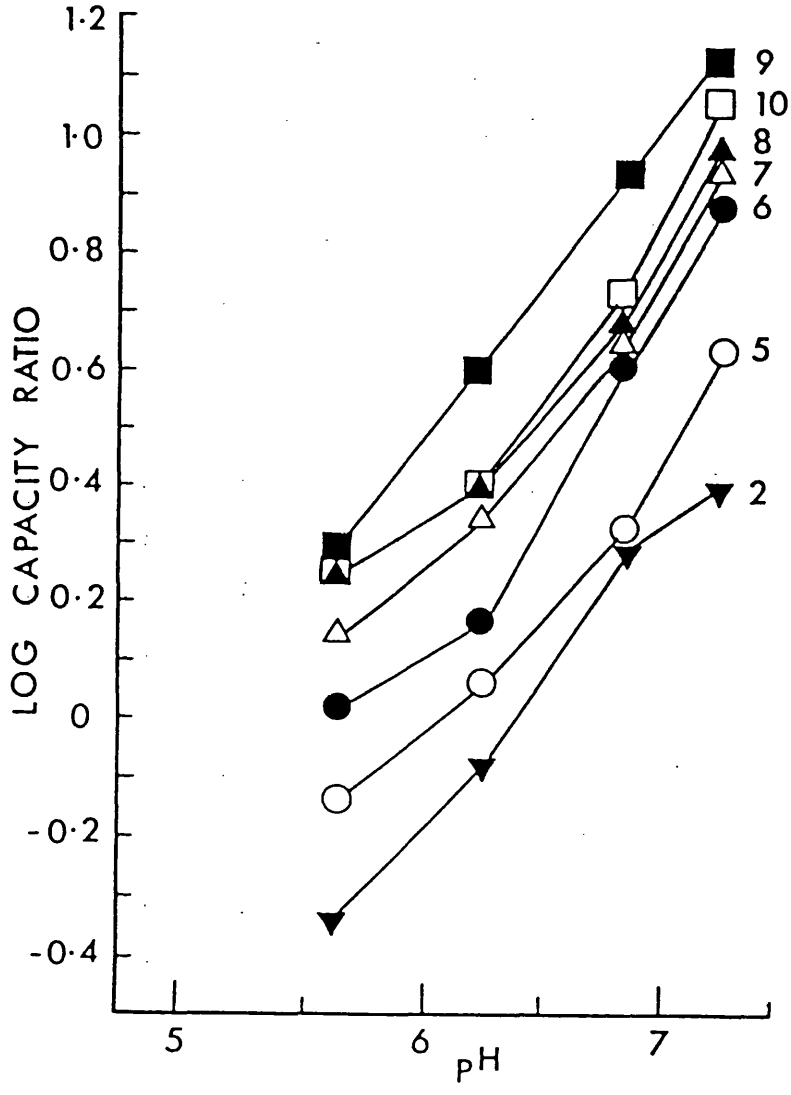


Figure 3.1. Solute codes as on opposite page.

Figure 3.2 Influence of mobile phase pH on the retention of some Group B compounds. Mobile phase, 0.005 M phosphate buffer-propan-2-ol-acetonitrile (65: 17.5: 17.5, v/v/v). Other conditions as in Fig. 3.1. Solute codes as on opposite page.

Codes and Literature pKa for Group B Compounds

No	Compound	pKa
11	Pethidine	8.70
12	Pipradrol	9.71
13	Benzphetamine	6.60
14	Fentanyl	7.34
15	Normethadone	9.20
16	Methadone	8.25-9.64
17	Norpipanone	8.50
18	Piritramide	8.5
19	Dipipanone	8.50-9.08

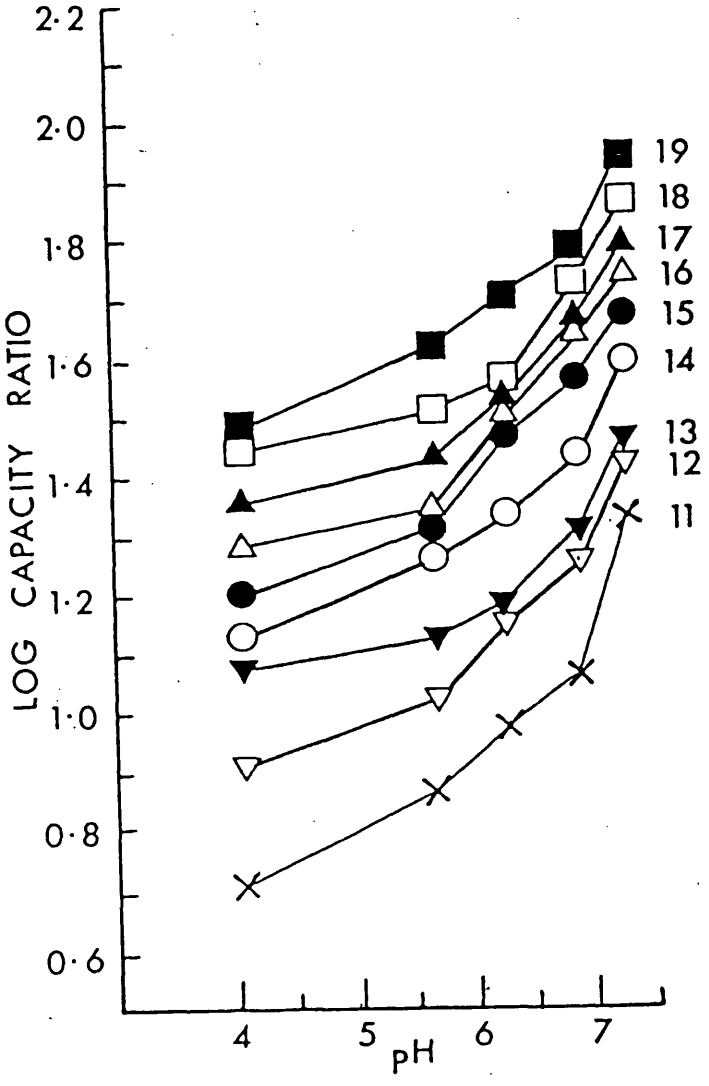


Figure 3.2.

Solute codes as on opposite page.

literature aqueous pKa values. However, considering that four of the compounds in Fig. 3.1 have pKa's between 9.6 and 10.4 (1), it was not expected that a total of 10% organic modifier would have such a marked effect on pKa. The relationship between solute retention, solute pKa and mobile phase pH was examined further, and is discussed in Section 4. For practical purposes, this study showed that the k' range could be varied between 0.5 and 2.0 at pH 5.4 and from 2 to 12 at pH 7.2.

A similar study was carried out for Group B solutes. Fig. 3.2 shows the same influence of mobile phase pH on solute retention as demonstrated in Fig. 3.1. The pH range was extended to 4.0 using 0.005 M KH_2PO_4 solution adjusted to pH by the addition of orthophosporic acid, and most solutes were eluted closer to their minimum k' values, i.e. the values expected when mobile phase pH is less than solute pKa -2. At this pH, protonation of the amines should be 100%. Since resolution was good at pH 4.0, this value was selected for further studies.

3.1.2b Influence of organic modifiers

At a buffer pH of 7.2 and a constant 5% v/v acetonitrile, the percentage of methanol in the mobile phase was increased stepwise up to 15% v/v for some Group A compounds. Figure 3.3 shows that solute log k'

values decreased linearly up to a point, however it will be noticed that the effect is greater on some drugs than others, but these differences are small and not an important consideration. From Table 3.1 which shows the effect of an increase in methanol concentration on methylamphetamine and two of its metabolites (p-hydroxyamphetamine and dexamphetamine), it could be observed that, gradually separation between them decreases, also phenmetrazine and phendimetrazine from Figure 3.3. It is of interest to note that the effect is quite significant on methylamphetamine, mephentermine and phendimetrazine - this could be due to the effect of a methyl group which is attached to the nitrogen atom, and this is common to all the three compounds. 10 percent v/v methanol was chosen for further investigation because it gave narrower peak shapes, reasonable k' values with fair resolution.

The effect of organic modifiers was also studied on Group B compounds and Figures 3.4 to 3.7 show that larger volumes of organic modifiers were required to achieve small k' values for these compounds because they are more hydrophobic than Group A compounds.

Recently it has been proposed that the solvent strength of a mobile phase may be measured by calculation of its partition coefficient, P_s (43). This has been suggested because in reversed-phase HPLC, the major influences that determine solute retention are

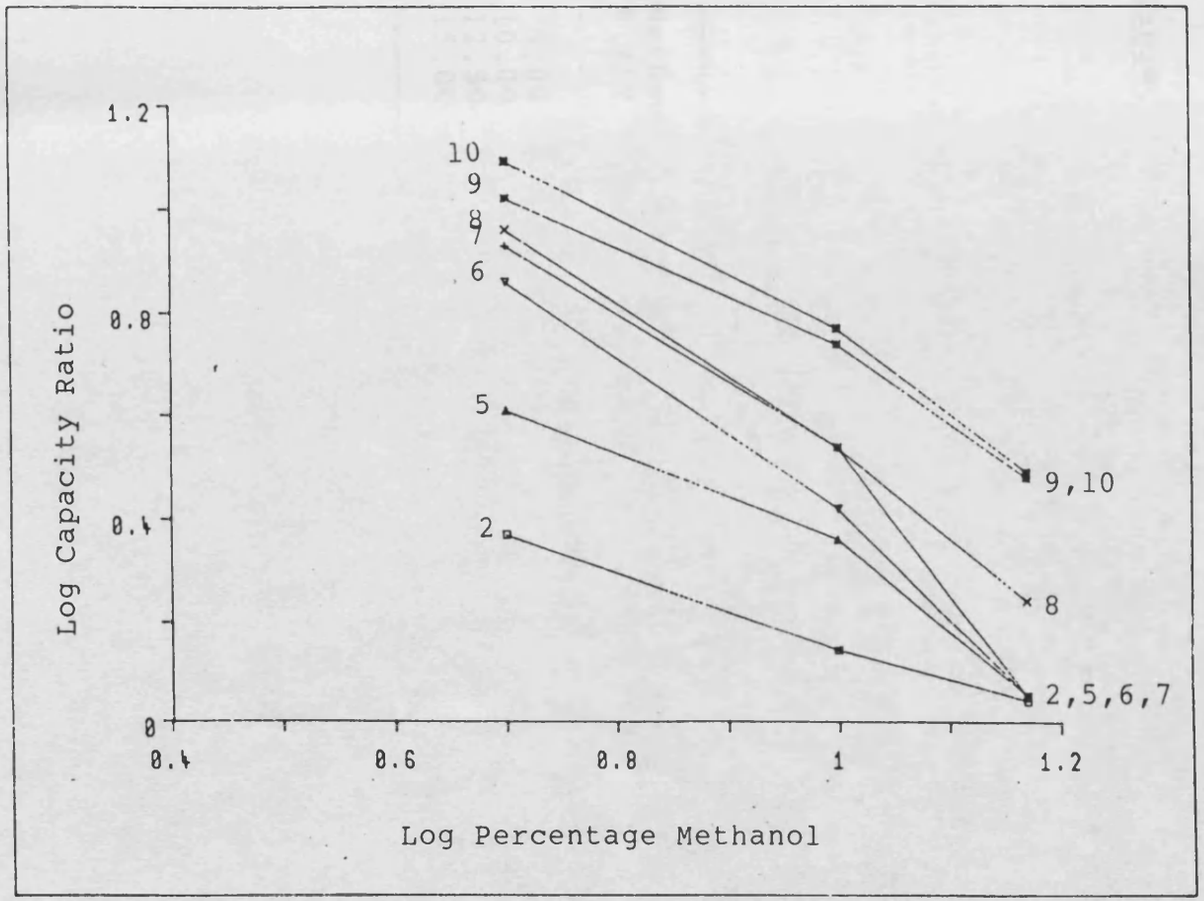


Figure 3.3 Influence of mobile phase methanol content on the retention of some Group A compounds. All other conditions as in Fig. 3.10.

Table 3.1. The relationship between the methanol concentration in the mobile phase and the capacity ratio (k') and selectivity (α) of p-hydroxyamphetamine (POHA), dexamphetamine (DEX) and methylamphetamine (METH). Chromatographic conditions as in Figure 3.1.

Methanol % v/v	POHA k'	POHA α	DEX k'	DEX α	METH k'
5.00	2.33	1.75	4.08	2.10	8.58
10.00	1.38	1.67	2.30	1.66	3.81
12.50	1.10	1.60	1.75	1.57	2.74
15.00	0.53	1.58	0.82	1.35	1.11

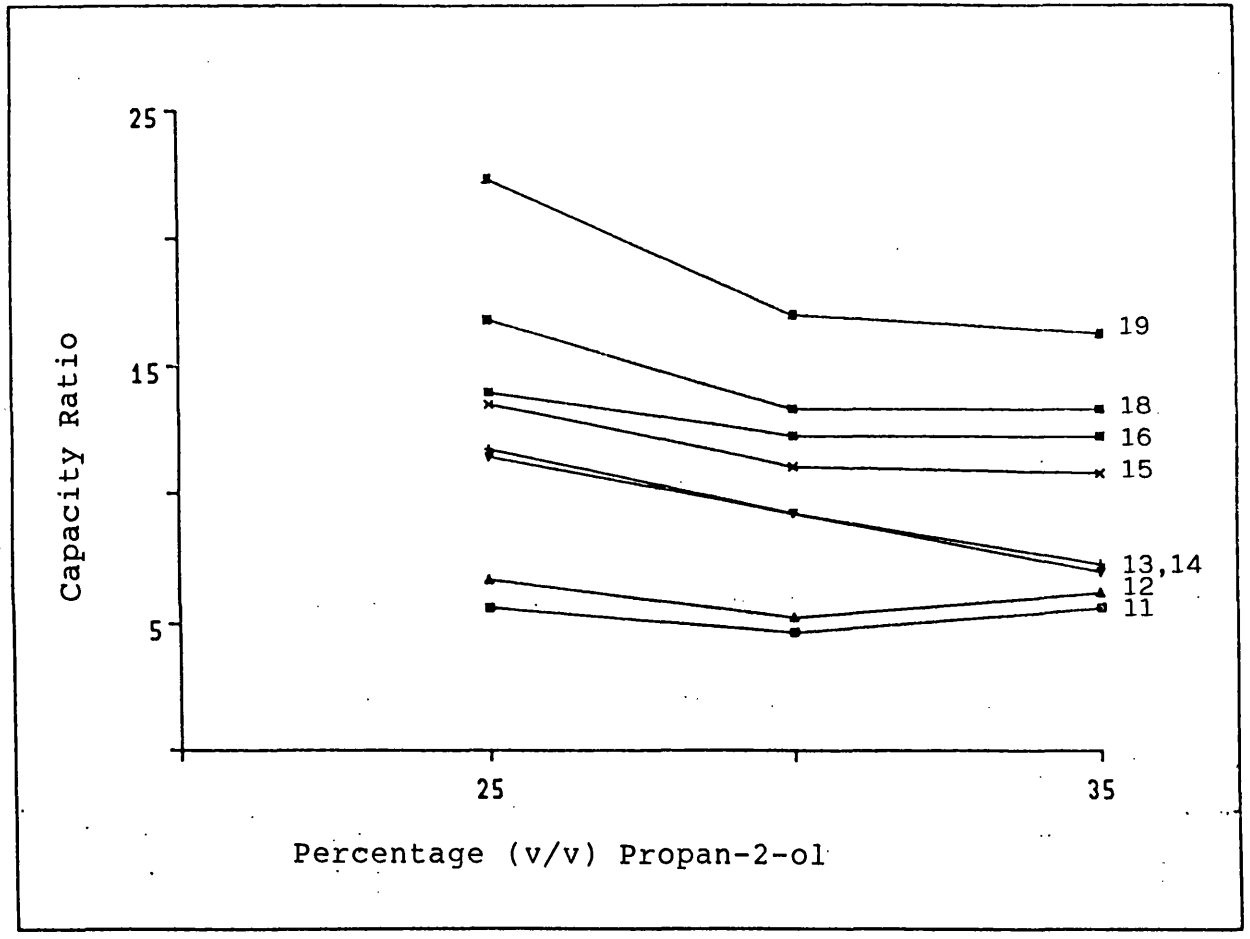


Figure 3.4 Influence of mobile phase propan-2-ol content on the retention of some Group B compounds. All other conditions as in Fig. 3.10.

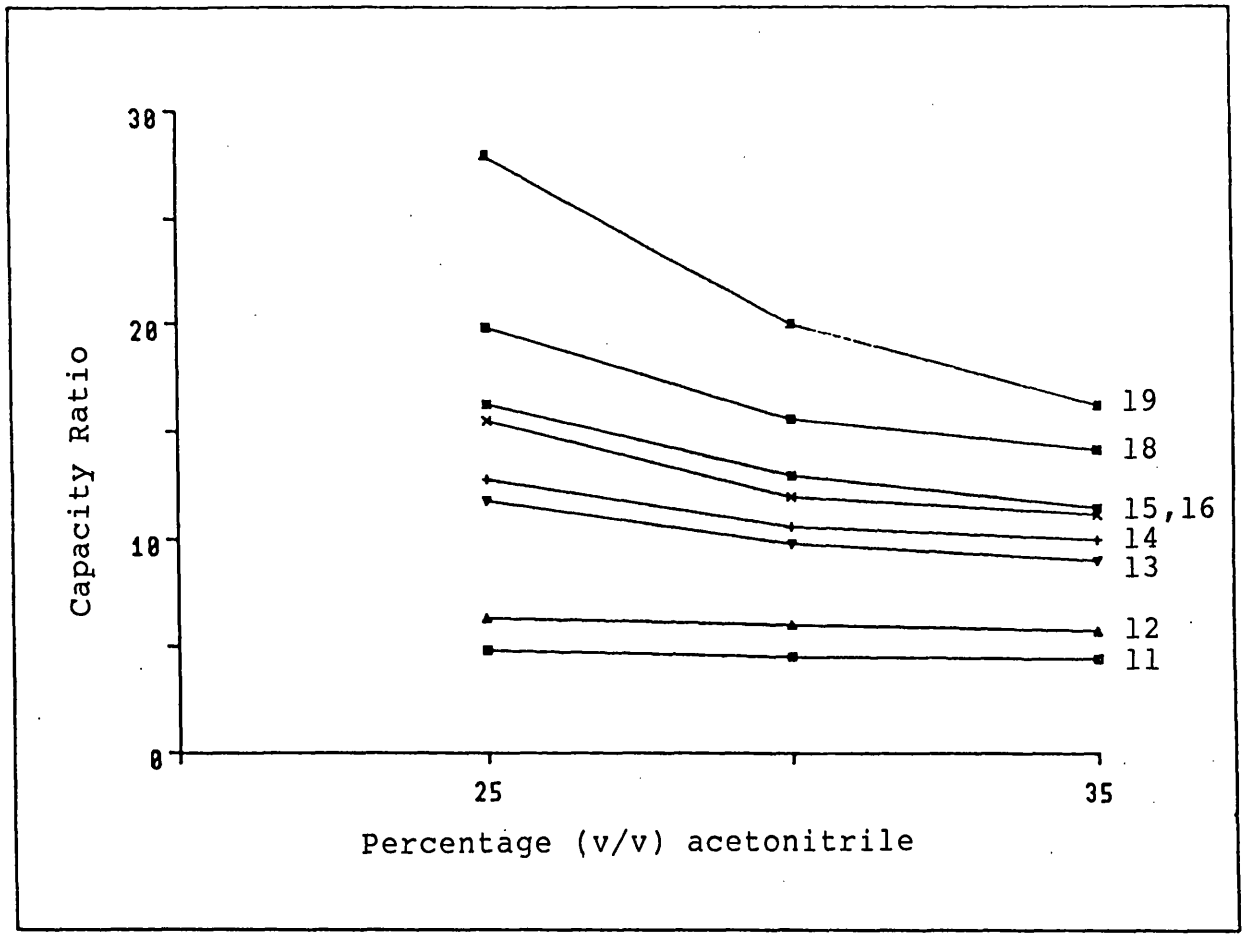


Figure 3.5 Influence of mobile phase acetonitrile content on the retention of some Group B compounds.

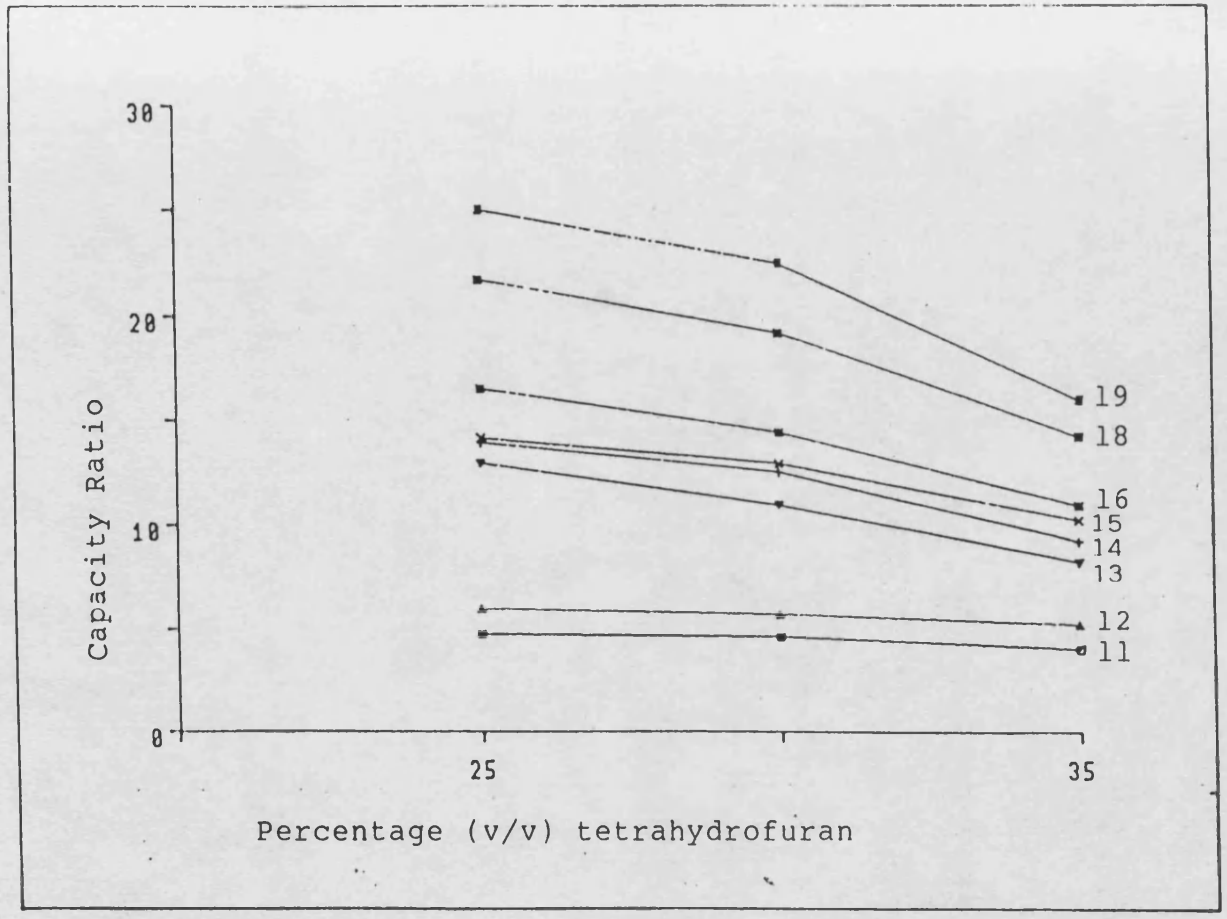


Figure 3.6 Influence of mobile phase tetrahydrofuran (THF) content on the retention of some Group B compounds.

Conditions for Figure 3.7

In all investigations 65% v/v 0.2 M phosphate buffer (pH 3.1) was used with:

A. 35% v/v Tetrahydrofuran (THF)

B. 35% v/v Propan-2-ol (Pr-2-OH)

C. 35% v/v Acetonitrile (ACN)

D. 17.5% : 17.5% v/v Pr-2-OH-ACN

E. 17.5%: 17.5% v/v THF-ACN

F. 17.5% : 17.5% v/v THF-Pr-2-OH

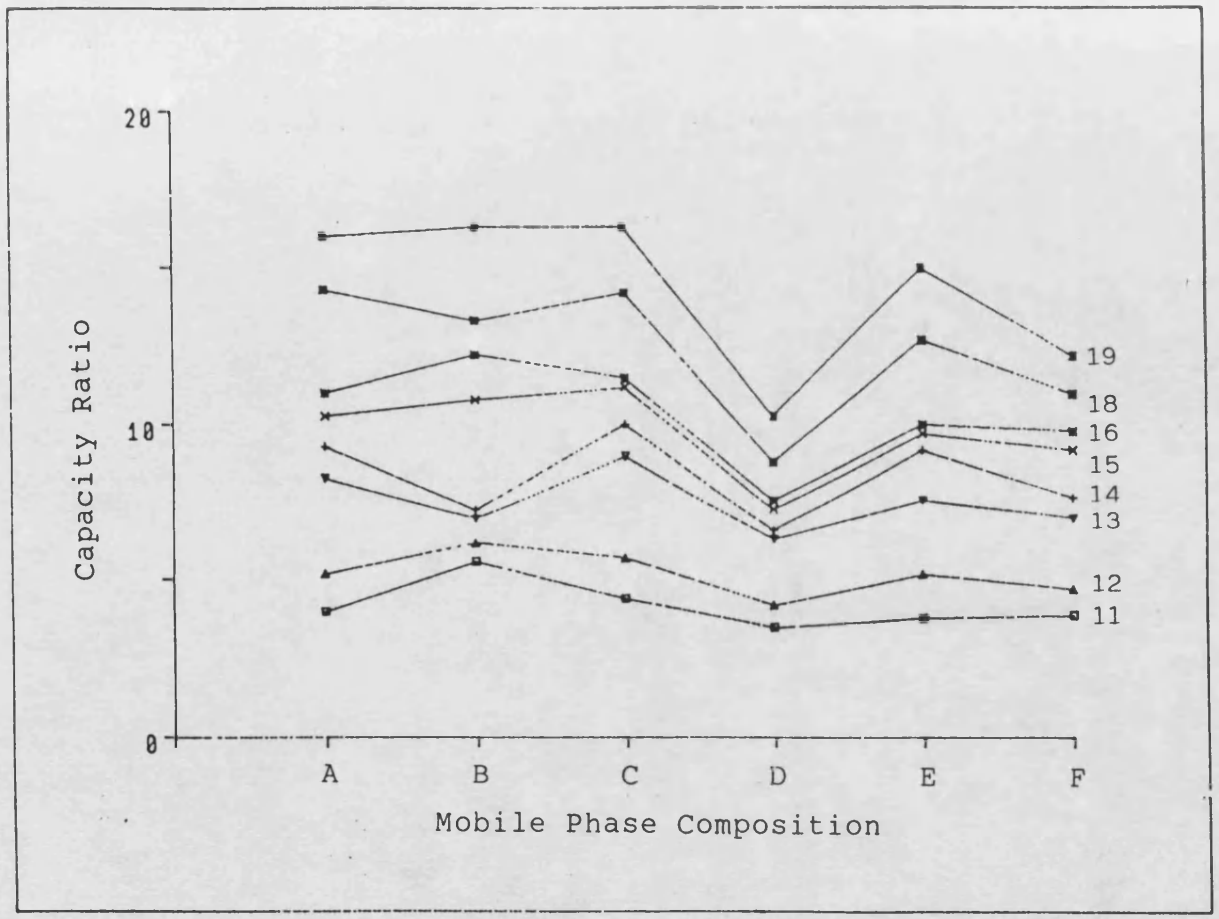


Figure 3.7 Effect of the combination of different organic solvent mixtures in the mobile phase on the retention of some Group B compounds. Solute codes as in Fig. 3.2. Mobile phase composition code as on opposite page.

the hydrocarbonaceous surface of the stationary phase and the solvent 'strength' of the mobile phase. When water is the mobile phase, then solute retention is dominated by the 'hydrophobic' effect and for aqueous-organic solvent mixtures it is the 'solvophobic' effect (44-48).

Solute retentions are also correlated to their n-octanol-water partition coefficients ($\log P$) (42), even though it is difficult to describe reversed-phase HPLC as a partition process. The partition coefficients of water, methanol, acetonitrile, propan-2-ol and tetrahydrofuran expressed as $\log P_s$ values are -1.38, -0.82, -0.34, +0.30 and +0.46 respectively (43).

The partition coefficient of a solvent mixture may be calculated as the sum of the mole fraction of each solvent present X, its $\log P_s$ value,

$$\text{namely } \log P_{sm} = \sum_{i=1}^{n} X_i . \log P_{si}$$

where m = mixture

X_i = mole fraction of ith solvent

$\log P_{si}$ = n-octanol-water log P for ith solvent

n = total number of solvents in the mixture.

The organic modifiers used in the optimization study have been examined using this approach to determine whether solute retentions may be reliably explained on this basis. The $\log P_s$ values for the single solvent-

water mixtures examined were calculated to be:

%	Pr-2-OH	ACN	THF
25	-1.258	-1.260	-1.255
30	-1.226	-1.246	-1.223
35	-1.191	-1.217	-1.187

The experimental k' values obtained with 35% of tetrahydrofuran and acetonitrile are very similar, having k' differences < ± 5%, whilst although propan-2-ol shows some selectivity differences, these are small and change k' by ±10%. The calculated partition coefficient values for these three solvent compositions are also very similar. However, when two organic modifiers are present with water then this approach does not explain the experimental k' values because they are lower than predicted. This is most pronounced whenever propan-2-ol is present, for example with propan-2-ol-acetonitrile- water (17.5; 17.5; 65% v/v/v), the value for log P_{sm} = -1.218, which is identical to that for acetonitrile- water (35; 65% v/v) and yet k' values are reduced by 25% (pethidine 5.8 to 4.2, dipipanone 16.2 to 10.2).

For propan-2-ol-tetrahydrofuran-water (17.5; 17.5; 65% v/v/v) the value for log P_{sm} = -1.203, which is similar to that for tetrahydrofuran-water (35; 65% v/v) at -1.187, but the k' values are reduced by 10

to 20% (pethidine 5.2 to 4.7, dipipanone 16.0 to 12.2).

It is likely that the different selective solute-solvent interactions of the solvents are partly responsible, because when two solvents are present together, then their solvating ability must be to some extent, additive. Another reason for the difficulty in applying the concept of partition coefficient as a solvent strength parameter in this particular case is that the concept was proposed following a study of the retention behaviour of unionized solutes. In this case the solutes are protonated amines at pH 4.0, in the presence of potassium dihydrogen phosphate and phosphoric acid.

It is likely that the amines are present in solution as ion-pairs with phosphate ions, so that their positive charge is partially neutralised. Furthermore this is a very different solute type from solutes such as benzonitrile, benzophenone and other similar compounds used in the log P_S study. The measurement of solvent strength using the Snyder Polarity index P' was also developed using unionised solutes and so is also unable to be used for the conditions described here. The concept of solvent selectivity as proposed by Rohrschneider (49) and Snyder (50) and then developed by Glajach et al. (51) into an optimization strategy is very helpful, and applicable to ionizable and unionizable solutes.

The relevant properties of the solvents used in this study are:

	X_e	X_d	X_n	p'
Pr-2-ol	0.55	0.29	0.27	3.9
THF	0.38	0.20	0.42	4.0
ACN	0.31	0.27	0.42	5.8

where Xe = proton acceptor contribution

Xd = proton donor contribution

Xn = strong dipole interaction

P' = solvent polarity

They show that THF and ACN have a similar emphasis on solute-solvent interactions, namely $X_n > X_e > X_d$ whereas the interactions of Pr-2-ol are $X_e > X_n > X_d$. This suggests that the optimum mixture for maximising the various solute-solvent interactions would be Pr-2-ol with either THF or ACN, and the experimental k' values in Fig. 3.7 show that retentions are reduced more when propan-2-ol is present. For a mobile phase at pH 4.0 with 0.005 M KH_2PO_4, 35% v/v of any one of these solvents produced very similar retentions in the k' range 4-16, with very little selectivity changes. When mixed in equal volumes propan-2-ol-acetonitrile reduced the k' range to 4-10, whilst propan-2-ol-THF gave 4-15. This suggests that no specific solute-solvent interactions sufficiently

important to influence retention order. It further suggests that hydrophobicity (log P) of solute is the most important property that influences the order of elution of these compounds under the above conditions. A chromatogram of Group B compounds under conditions selected for minimum, reproducible k' values is given in Fig. 3.10b, and shows good selectivity and sensitivity (12 ng each solute on-column).

3.1.2c Influence of Buffer Concentration

The influence of buffer molarity on solute retention was studied over the range 0.005 M to 0.05 M. Retention was reduced for all solutes with increase in buffer concentration, Figure 3.8. The range 0.025-0.05 M was preferred because it would routinely produce more reproducible k' values due to the relatively small decrease in these values in this range. It is probable that phosphate ions form ion-pairs with protonated solutes, especially since at pH 7.2 HPO_4^{2-} ions rather than $H_2PO_4^-$ ions, predominate. The uncertainty surrounding pKa values in the presence of organic modifier makes the estimation of this effect difficult, although the results are of practical value. A chromatogram of the ten compounds in Group A optimised for pH, buffer and organic solvents show good resolution and sensitivity (6-12 ng each solute, on-column) within an analytical time of eight

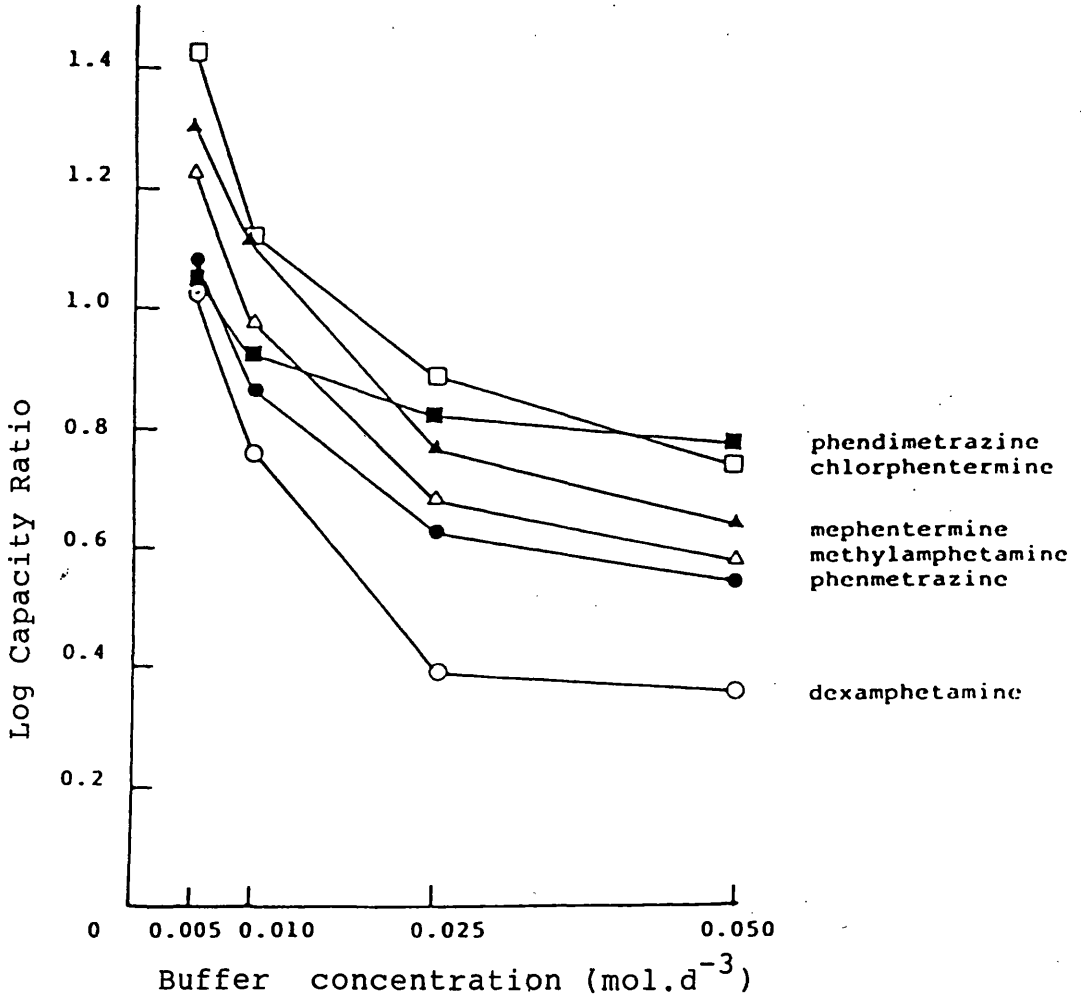


Figure 3.8 Influence of mobile phase buffer ionic strength on some Group A compounds. Mobile phase pH was 5.4.

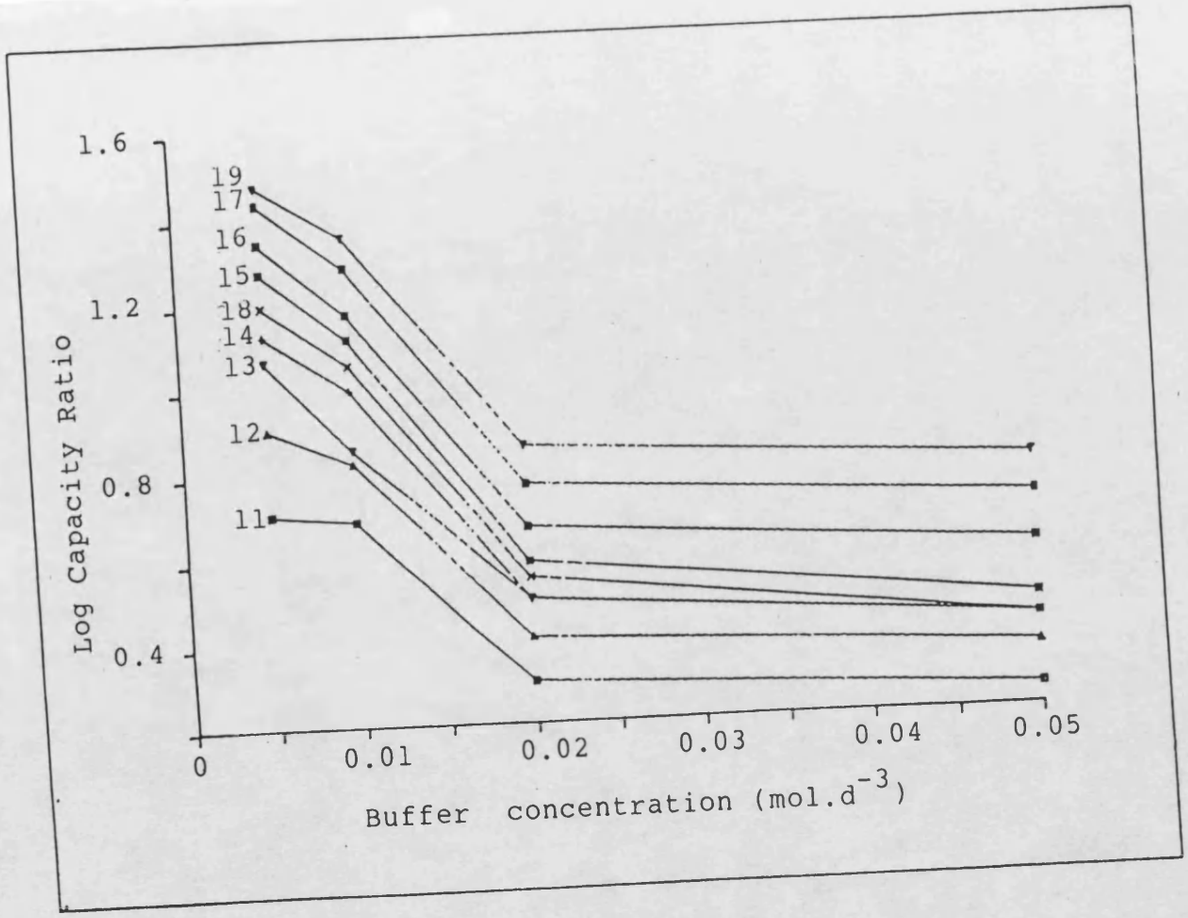


Figure 3.9 Influence of mobile phase buffer ionic strength on the retention of some Group B compounds.

Conditions for Figure 3.10(a)

5 - µm CPS - Hypersil column (50 x 4.6 mm.i.d) with 0.025 M Phosphate buffer (pH 7.2) - methanol-acetonitrile (90:5:5, V/V/V) at 30°C, 1 ml.min.$^{-1}$ flowrate monitored at 205 nm, 0.04 a.u.f.s.

Injection volume (10 µl) contained 6-12 ng of each solute.

Conditions for Figure 3.10(b)

Same as fig 3.10(a) but with a mobile phase of 0.02 M Phosphate buffer (pH 4.0) - propan-2-ol-acetonitrile (65:17.5:17.5, V/V/V).

Injection volume (10 µl) contained about 12 ng of each solute.

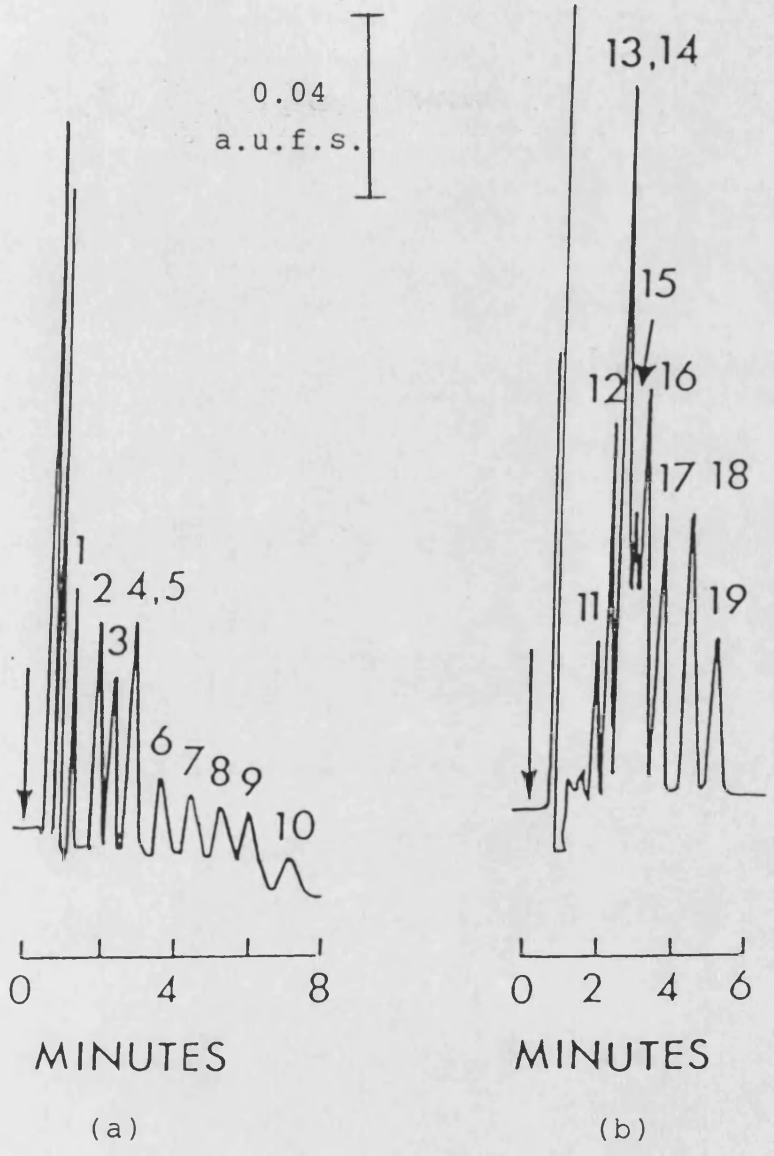


Figure 3.10 Chromatograms of Group A (a) and Group B (b) compounds. Conditions as on opposite page. Solute codes as in Figures 3.1 and 3.2 respectively.

minutes, Figure 3.10a.

The influence of buffer molarity on solute retention for Group B compounds, showed the same relationship as for Group A. It is interesting that for six solutes in Group A, retentions at 0.005 M were in the range log k' 1.0 - 1.4 and fell to log k' 0.4 - 0.8 at 0.05 M whilst seven solutes in Group B having a log k' range of 1.05 - 1.48 at 0.005 M similarly fell to log k' 0.4 - 0.8 at 0.05 M buffer, Figure 3.9. Considering the mobile phase pH was 7.2 for Group A and 4.0 for Group B, this suggests that ion-pair formation between protonated solute and phosphate anions cannot be the main factor in the effect that buffer has upon solute retention. The solute pKa range in Group A (6.8 - 10.4) is very similar to the seven solutes considered in Group B (6.6 - 9.2).

3.1.3 Quantitative Aspect

The suitability of the procedures obtained for cyanopropyl-bonded silica for quantitative determinations was examined using a 50 x 4.6 mm i.d. column (conditions as described in Figure 3.10). Six-point calibrations were made for dexamphetamine, phenmetrazine, methylamphetamine and mephentermine in mobile phase, over the concentration 0.25 - 5 $\mu g\ ml^{-1}$. The results are shown in Table 3.2 and Figures 3.11a-3.11d. Linear relationships were obtained between

Table 3.2. Statistical analysis of results for the calibration of dexamphetamine (DEX), phenmetrazine (PHEN), methylamphetamine (METH) and mephentermine (MEPH) and calculation of percentage recovery from plasma. Conditions as in Figure 3.10a.

	DEX		PHEN		METH		MEPH	
	Control	Plasma	Control	Plasma	Control	Plasma	Control	Plasma
Correlation coefficient	1.00	0.996	0.999	0.999	1.00	1.00	0.999	0.977
Slope	78.30	61.44	59.87	51.47	139.58	137.93	75.00	61.10
Std. dev. of slope	0.2	2.02	0.565	0.744	0.25	0.62	1.40	0.11
RSD of slope (±) %	0.512	5.652	1.58	2.07	0.52	1.29	3.58	0.33
Percentage recovery		80.0		86.3		98.7		81.1
Precision (n=10)								
Mean (peak heights) (mm)		29.1		49.4		68.7		64.6
Std. dev.		0.994		2.16		0.95		
RSD (±, %)		3.4		4.4		3.8		1.31

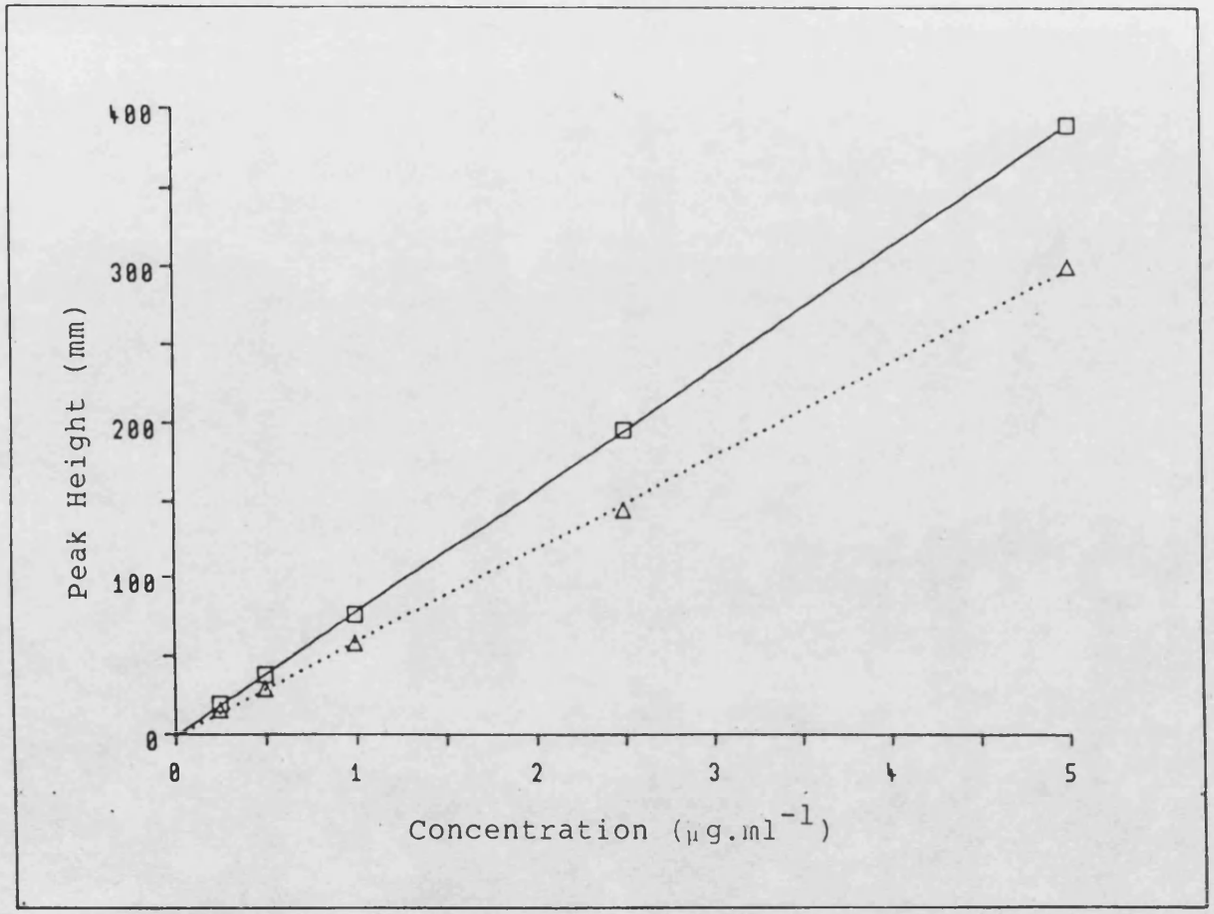


Figure 3.11a Calibration of dexamphetamine (0.25-5.0 µg.ml^{-1}).
Control (——) and extract from spiked plasma (.....)
Conditions as in Fig. 3.10a.

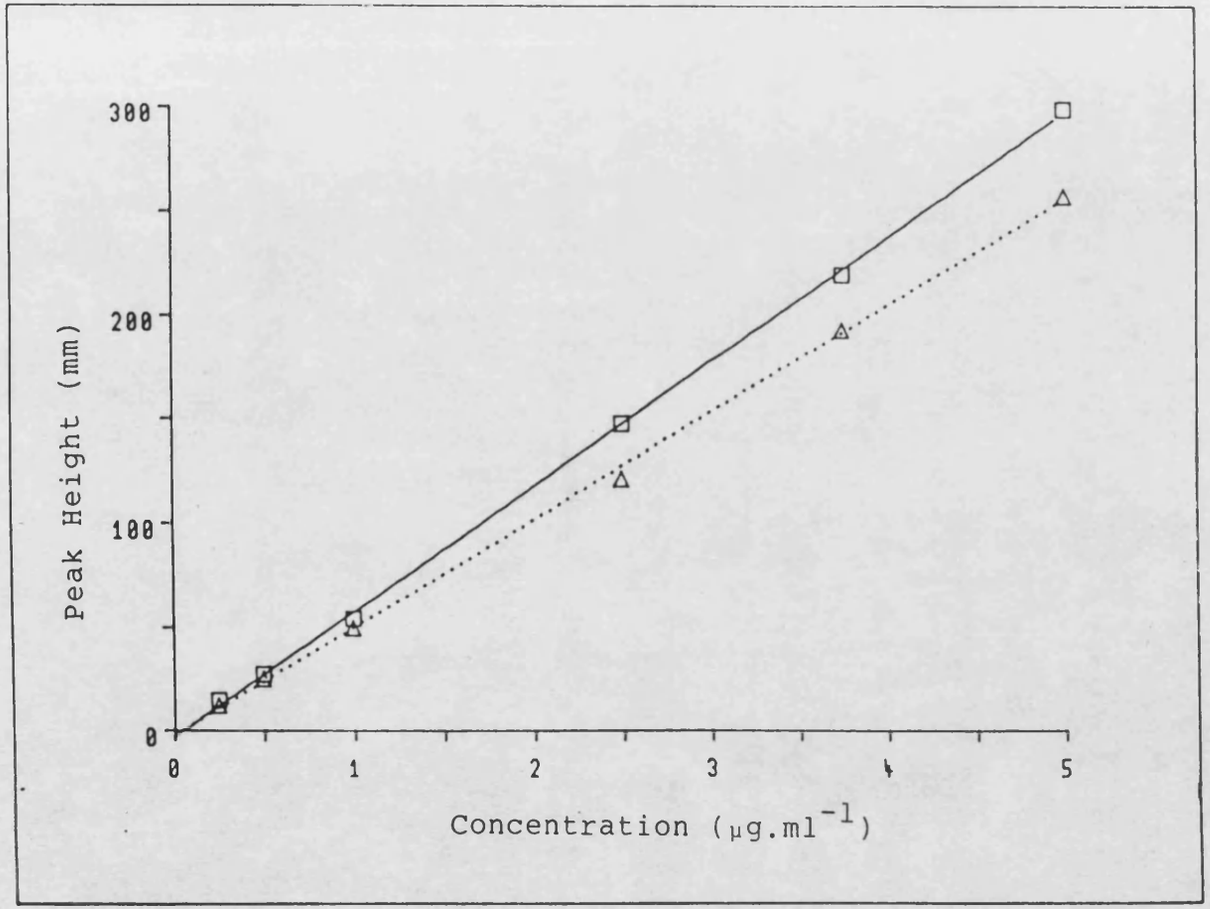


Figure 3.11b Calibration of phenmetrazine (0.25-5.0 µg.ml^{-1}). Control (——) and extract from spiked plasma (·····). Conditions as in Fig. 3.10a.

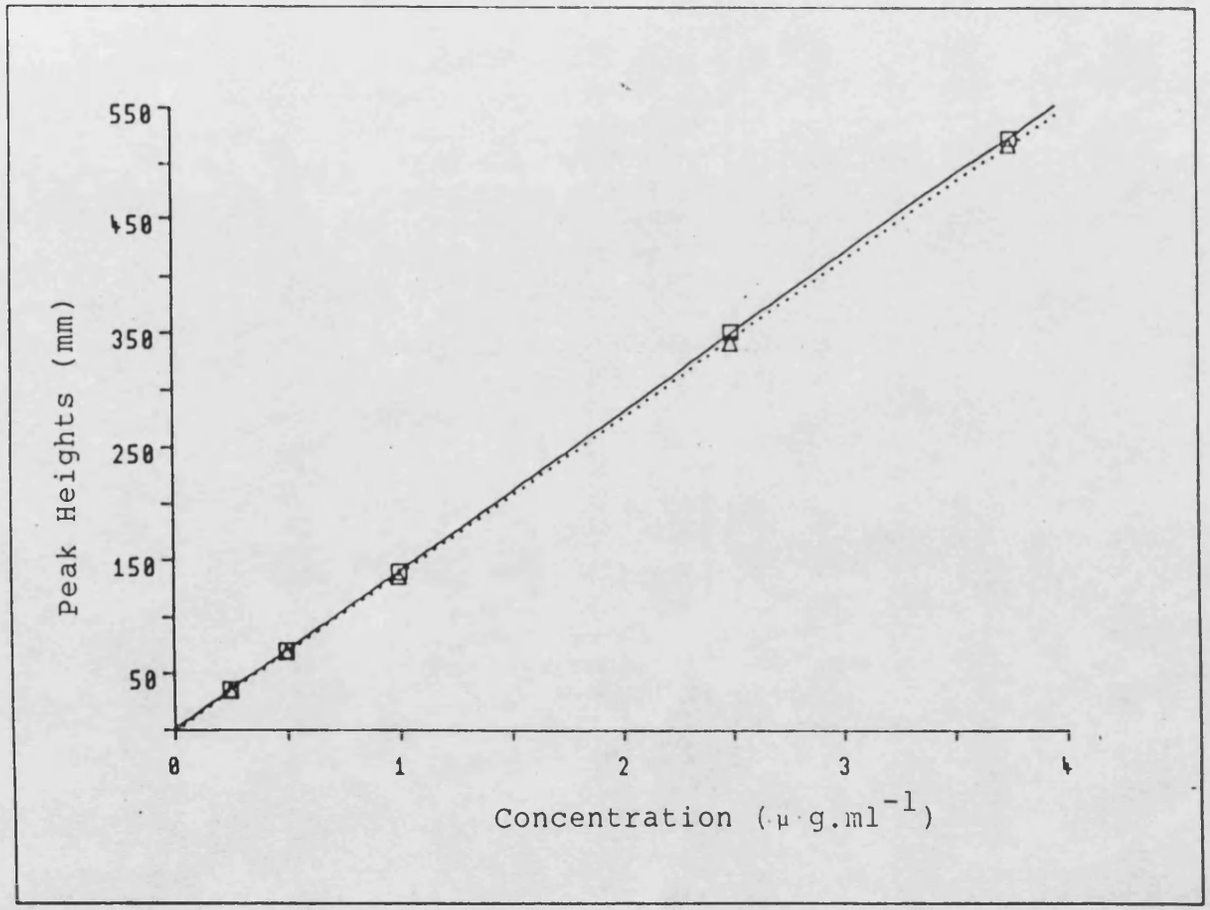


Figure 3.11c Calibration of methylamphetamine (0.25-3.75 μg.ml^{-1}), Control (——) and extract from spiked plasma (·····). Conditions as in Fig. 3.10a.

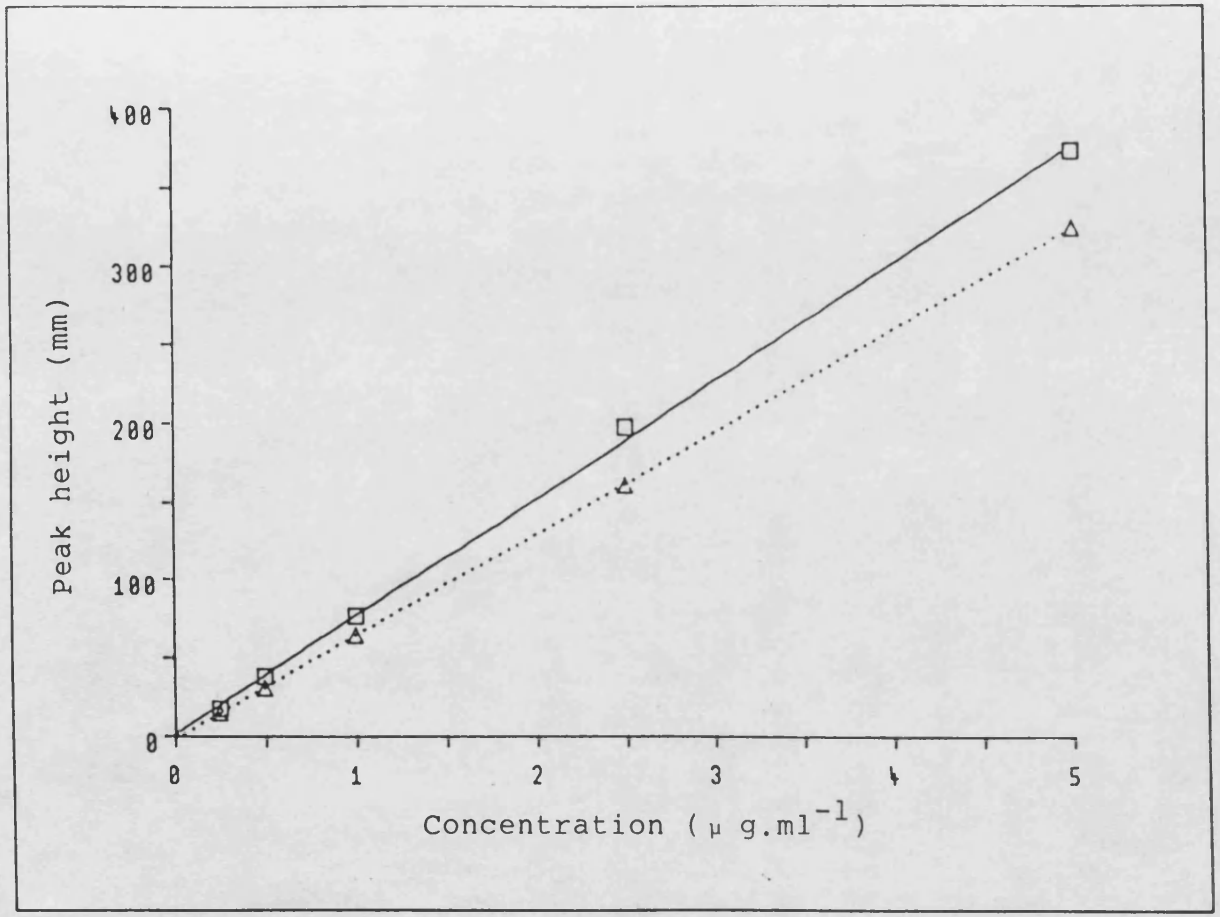


Figure 3.11d Calibration of mephentermine (0.25-5.0 μg.ml^{-1}), Control (——) and extract from spiked plasma (····). Conditions as in Fig. 3.10a.

peak height and concentration of solute for duplicate injections, with intercepts not significantly different from zero. Correlation coefficients for each calibration were > 0.999 and relative standard deviations (RSD) for the slopes were ± 0.32, 0.94, 1.9 and 0.41% respectively. Identical calibrations were made by spiking plasma with the same stock calibration solutions, followed by their extraction as described in Section 2.4. Linear relationships were obtained as before, with intercepts not significant; correlation coefficients were 0.996, 0.999, 0.977 and 0.999 respectively. The percentage recovery of solutes from plasma, calculated as the spike calibration slope/control calibration slope, x100 was found to be 80.8, 86.3, 81.1 and 98.7% respectively. All these calibrations had been made without the benefit of an internal standard in order to test the quantitative behaviour of each solute. The calibrations show this to be satisfactory, permitting any one solute to be used as an internal standard for the other solutes.

This quantitative aspect was extended to a Group B compound and its metabolite (methadone and normethadone) with dipipanone as the Internal Standard using the chromatographic conditions described in Figure 3.10b and the statistical results are shown in Table 3.3 and Figures 3.12a-3.13b. Concentration range was 0.25-5.0 $\mu g.ml^{-1}$. The calibrations were carried out

Table 3.3. Statistical analysis of normethadone and methadone (0.25-5.0 $\mu g.ml^{-1}$) using dipipanone as internal standard. Chromatographic conditions as in Figure 3.10b.

	NORMETHADONE				METHADONE			
	PEAK HEIGHT		PEAK HEIGHT RATIO		PEAK HEIGHT		PEAK HEIGHT RATIO	
	Control	Plasma	Control	Plasma	Control	Plasma	Control	Plasma
Correlation coefficient	0.9992	0.9996	0.9999	0.9994	0.9998	0.9998	0.9998	0.9996
Slope	82.49	67.69	0.518	0.444	74.79	59.85	0.469	0.394
Std. dev. of slope	1.69	1.09	0.0039	0.0075	0.69	0.715	0.0051	-0.0055
Intercept	6.98	6.60	0.014	0.0079	3.546	-0.88	-0.004	-0.004
Std. dev. of intercept	4.71	2.82	0.011	0.021	1.933	1.994	-0.014	-0.0152
RSD of slope (±), %	2.05	1.61	0.75	1.69	0.92	1.19	1.09	1.40
Percentage recovery(%)		82.11		85.71		80.33		84.01
Precision (n=10)								
Mean peak heights (mm)	88.5	67.1			77.7	56.2		
Std. deviation	1.20	1.45			1.50	1.17		
RSD (±, %)	1.36	2.20			1.90	2.10		

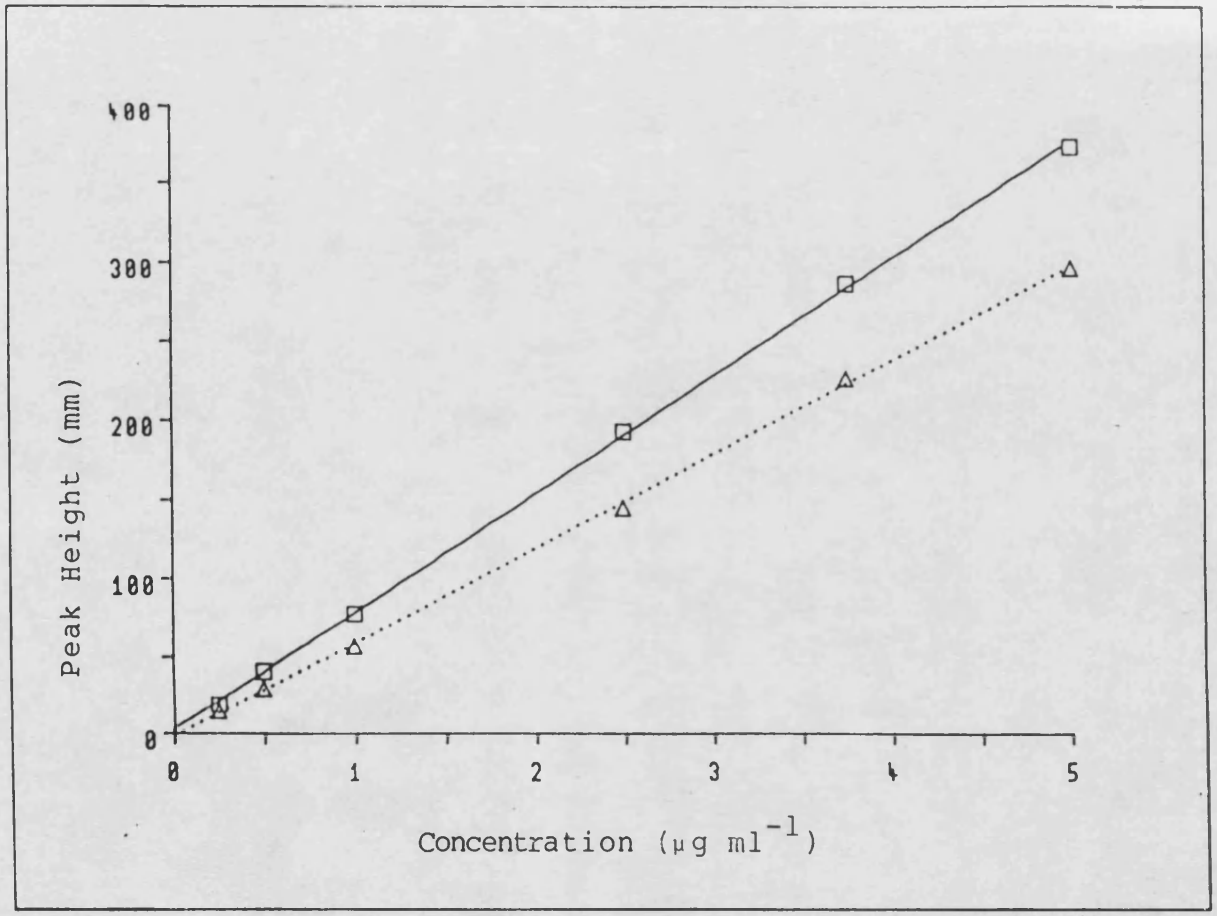


Figure 3.12a Calibration of methadone, using peak heights (mm) 0.25-5.0 µg.ml^{-1}). Control (——) and extract from spiked plasma (····). Conditions as in Figure 3.10b.

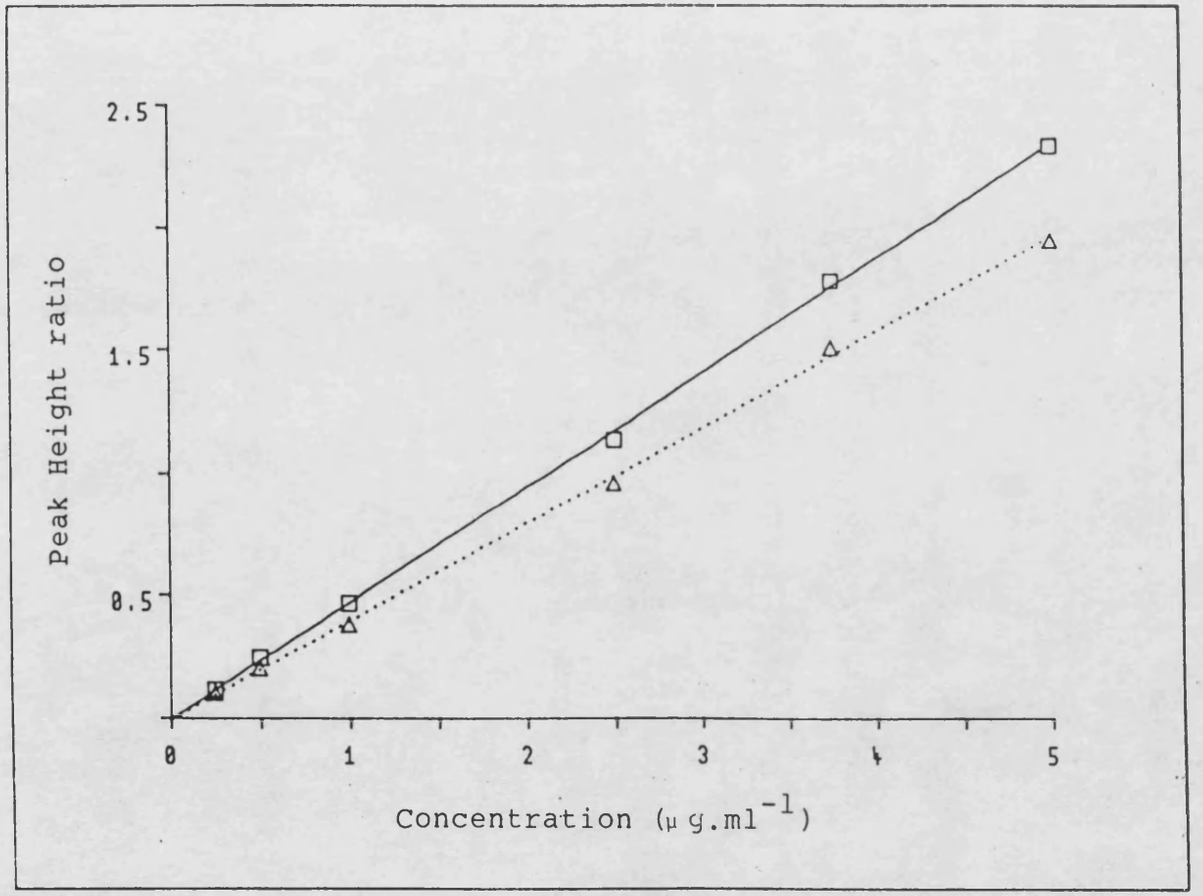


Figure 3.12b Calibration of methadone, using peak height ratios.

All other conditions as in Figure 3.10b.

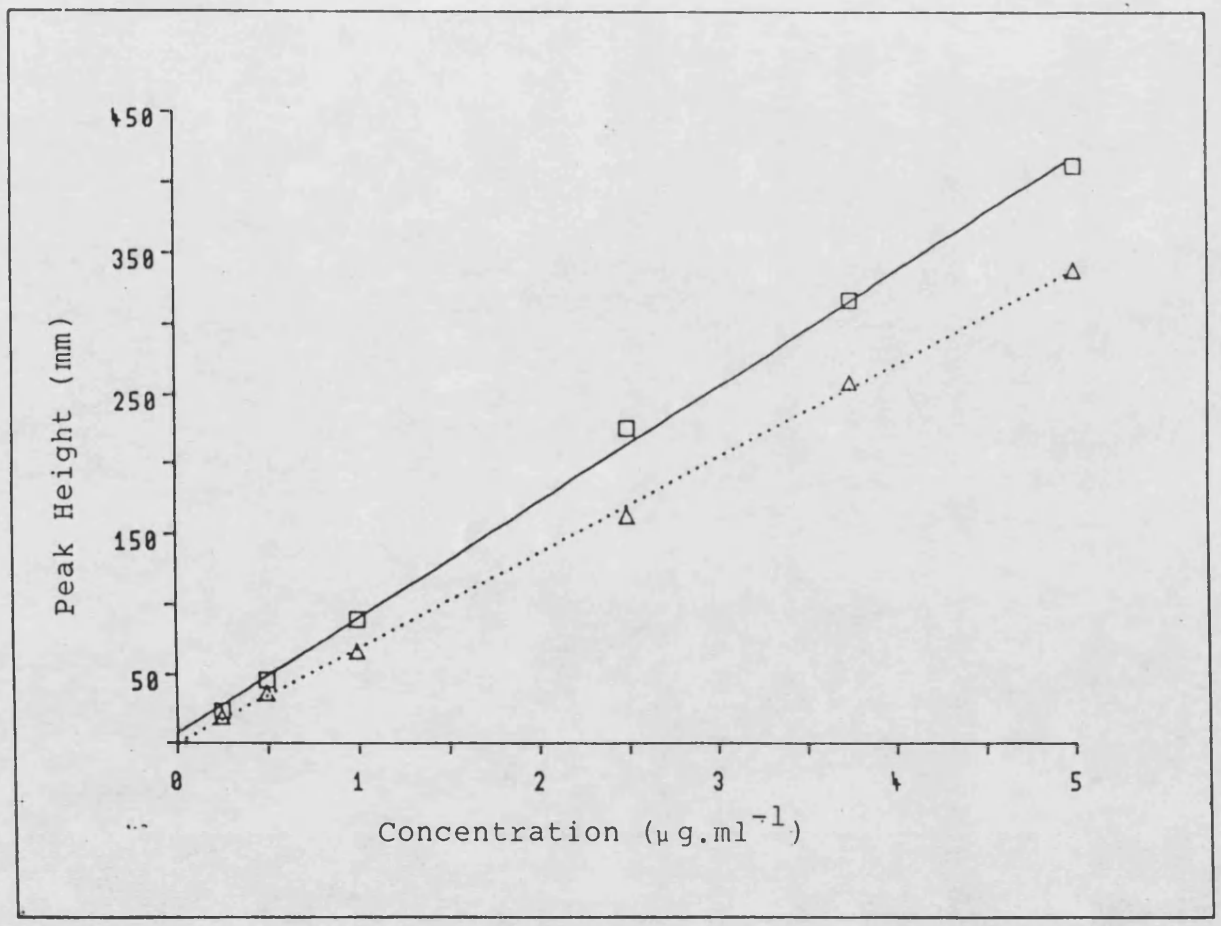


Figure 3.13a Calibration of normethadone using peak heights (mm) (0.25-5.0 $\mu g.ml^{-1}$), control (——) and extract from spiked plasma (····). Conditions as in Fig. 3.10b.

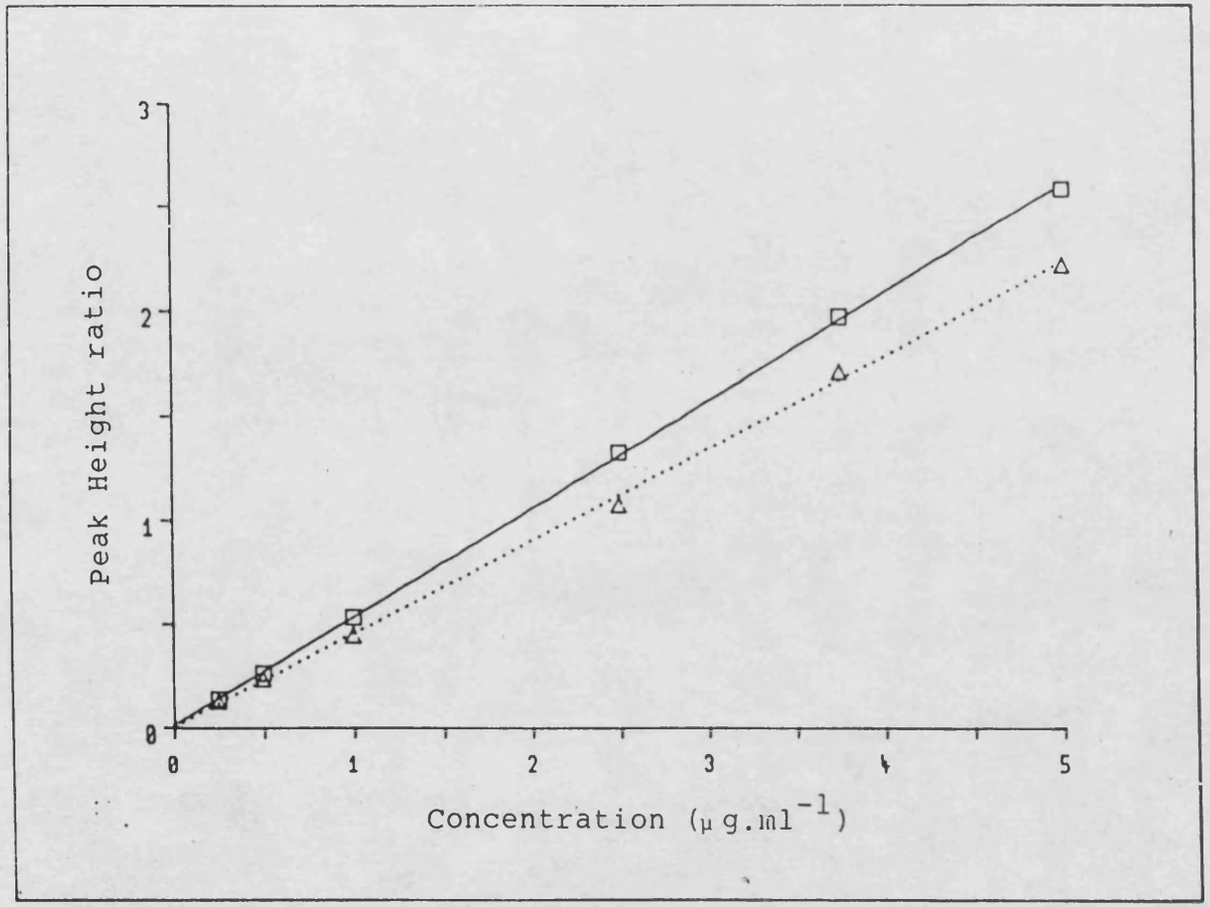


Figure 3.13b Calibration of normethadone, using peak height ratios.

All other conditions as in Figure 3.10(b).

using both peak heights and peak height ratios, for comparison and to check the suitability of the system. Linear relationships were also obtained in all cases. With peak heights against concentration correlation coefficients for both methadone and normethadone were 0.999 and relative standard deviations for the slopes were ± 0.92% and ± 2.05% respectively. For identical calibration of extracted spiked plasma with the same stock calibration solutions, correlation coefficients were > 0.999 and relative standard deviations were ± 2.1% and ±2.2% respectively. In all cases the intercepts were not significant. When peak heights were used for calibrations, correlation coefficients were > 0.999 for both control and plasma extract and relative standard deviations were ± 1.90% and ±1.36% respectively.

In order to make the method more readily applicable to other workers, a commercially available 100 x 2.1 mm i.d. cartridge column containing 5 μm cyanopropyl-silica was tested for Group B compounds. The column temperature was raised to 40°C in order to reduce the organic modifier content from 35% to 24%, thus reducing the background noise (absorbance) at 205 nm. Figure 3.14 shows that good resolution was obtained with similar capacity factors (1.5-9.0) and column efficiency (N = 900-1350) to those in Figure 3.10b, although there was slight tendency for some peak asymmetry (AS = 1.0-1.3), Table 3.4. Six point

Figure 3.14 Chromatogram of the separation of Group B compounds. Conditions: 5 μm Spheri-Cyano cartridge column (100 x 2.1 mm i.d.) with a mobile phase of 0.025 M phosphate buffer - propan-2-ol - acetonitrile (76: 12: 12, v/v/v) at 40°C. Injection volume (10 μl). Flow rate was 0.5 ml.min^{-1}, monitored at 205 nm with 0.04 a.u.f.s. Compounds as in Figure 3.10b.

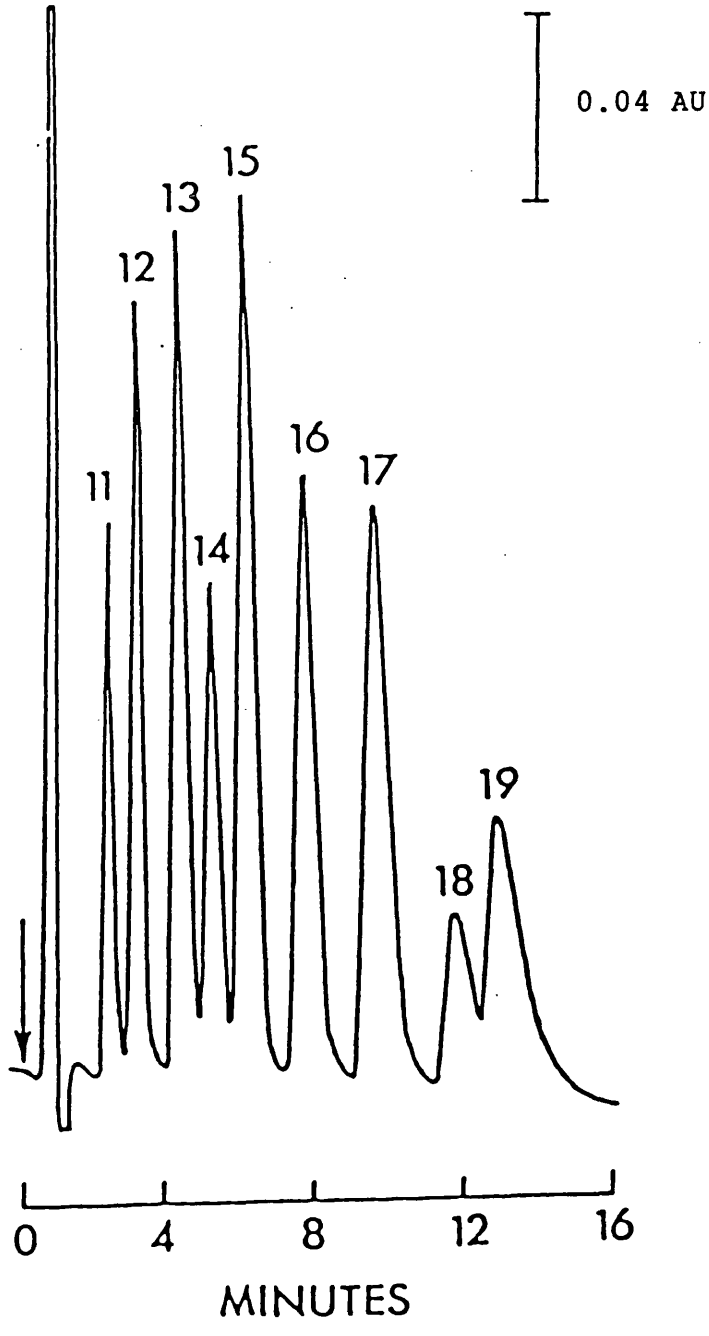


Figure 3.14

Table 3.4. Summary of the capacity ratio (k'), resolution (R_s), column efficiency (N) and peak asymmetry (As) of some Group B compounds. Conditions as in Figure 3.14.

Compound	k'	N	As	R_s
Pethidine	1.4	798	1.0	
				3.6
Benzphetamine	3.4	1086	1.3	
				2.3
Normethadone	5.2	1165	1.35	
				1.6
Methadone	6.8	1357	1.17	
				1.6
Norpipanone	8.75	1219	1.3	
				1.9
Dipipanone	12.1	952	2.3	

calibrations were made for a mixture of pethidine, benzphetamine, normethadone, methadone and dipipanone, over the concentration range 0.7-18 $\mu g\ ml^{-1}$ using norpipanone as internal standard (Figure 3.15). Linear relationships between peak area ratios and solute concentration were obtained (Figure 3.16) with correlation coefficients >0.998 and intercepts not significant. The precision (n = 7) for each solute solution gave relative standard deviations of ±1.45, 1.27, 0.87, 0.55 and 4.7% respectively at the 9 $\mu g\ ml^{-1}$ level, results are shown in Table 3.5.

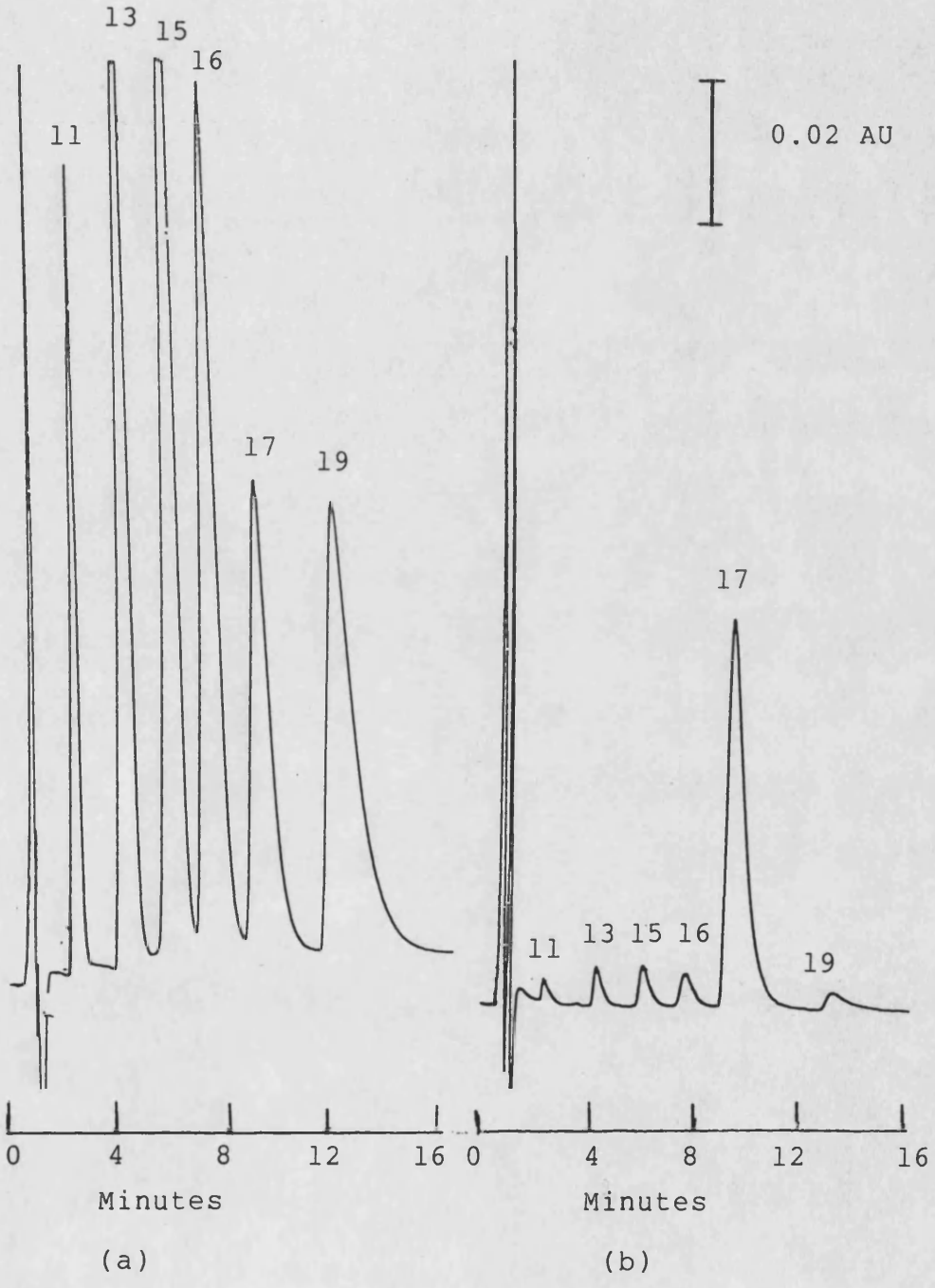


Figure 3.15. Chromatograms of highest (a) and lowest (b) concentration for the calibration of some Group B compounds.Conditions as in Figure 3.14.

Table 3.5. Statistical analysis of the calibration of some Group B compounds (0.7-18 $\mu g.ml^{-1}$), using norpipanone as internal standard and peak area ratios. Conditions as in Figure 3.15.

	Pethidine	Benzphetamine	Normethadone	Methadone	Dipipanone
Correlation	0.999	0.9993	0.9996	0.9993	0.9984
Slope	0.0325	0.0742	0.098	0.0880	0.0804
Std. dev. of slope	0.0005	0.0014	0.0014	0.0016	0.0026
Intercept	0.0037	0.00089	-0.00009	-0.0052	0.0191
Std. dev. of intercept	0.005	0.012	0.0119	0.0136	0.024
Variance	0.044	0.1978	0.3451	0.2784	0.2623
Precision (n)	n = 4	n = 7	n = 7	n = 7	n = 7
Mean (Peak area ratios)	0.3039	0.6973	0.9114	0.8173	0.7804
Standard deviation	0.0044	0.00887	0.00791	0.00448	0.0368
Relative standard deviation	1.45%	1.27%	0.87%	0.55%	4.7%
Variance	0.000015	0.000067	0.000054	0.000017	0.00116

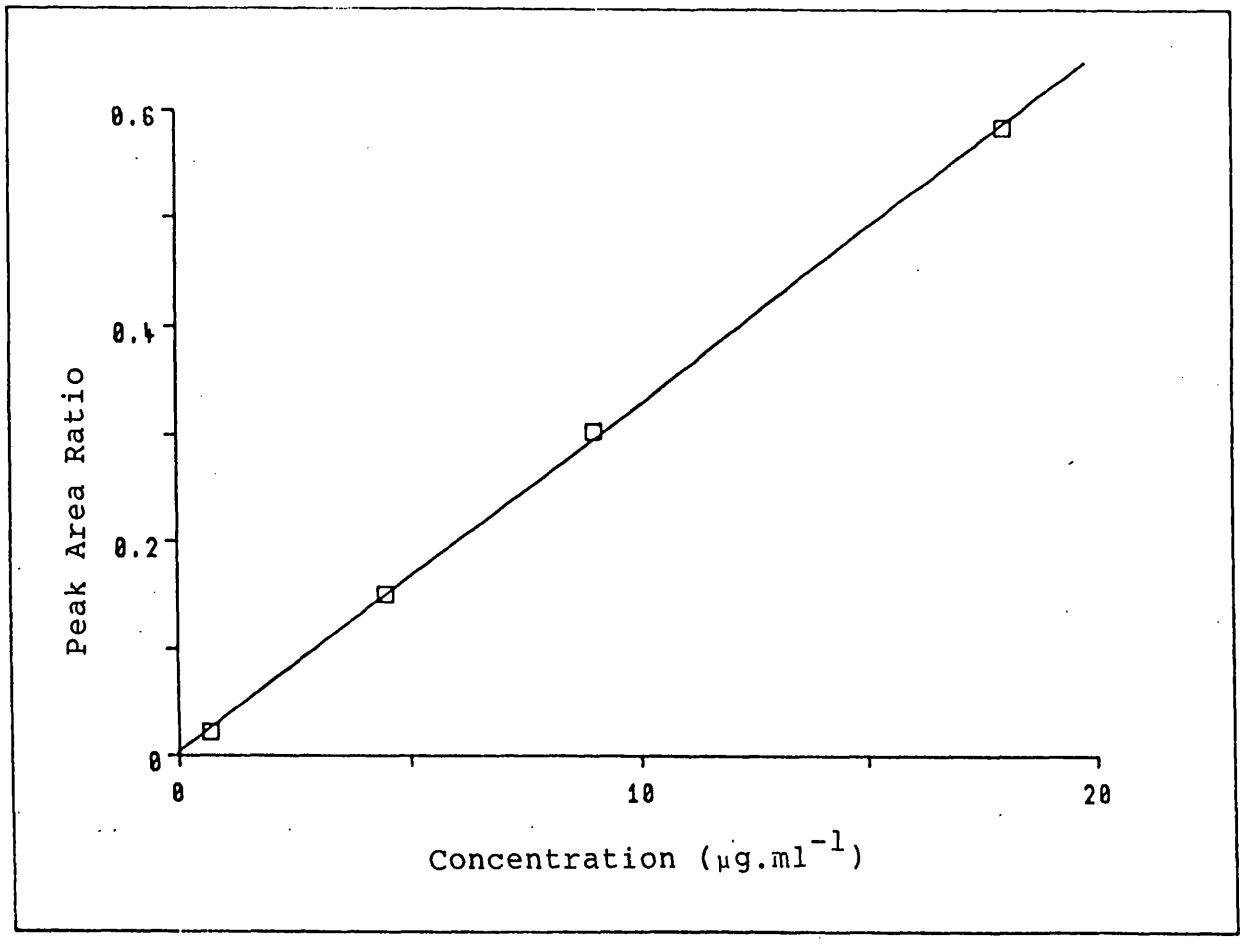


Figure 3.16a Calibration of pethidine (0.7-18 µg.ml^{-1}) with norpipanone as internal standard. Conditions as in Fig. 3.15.

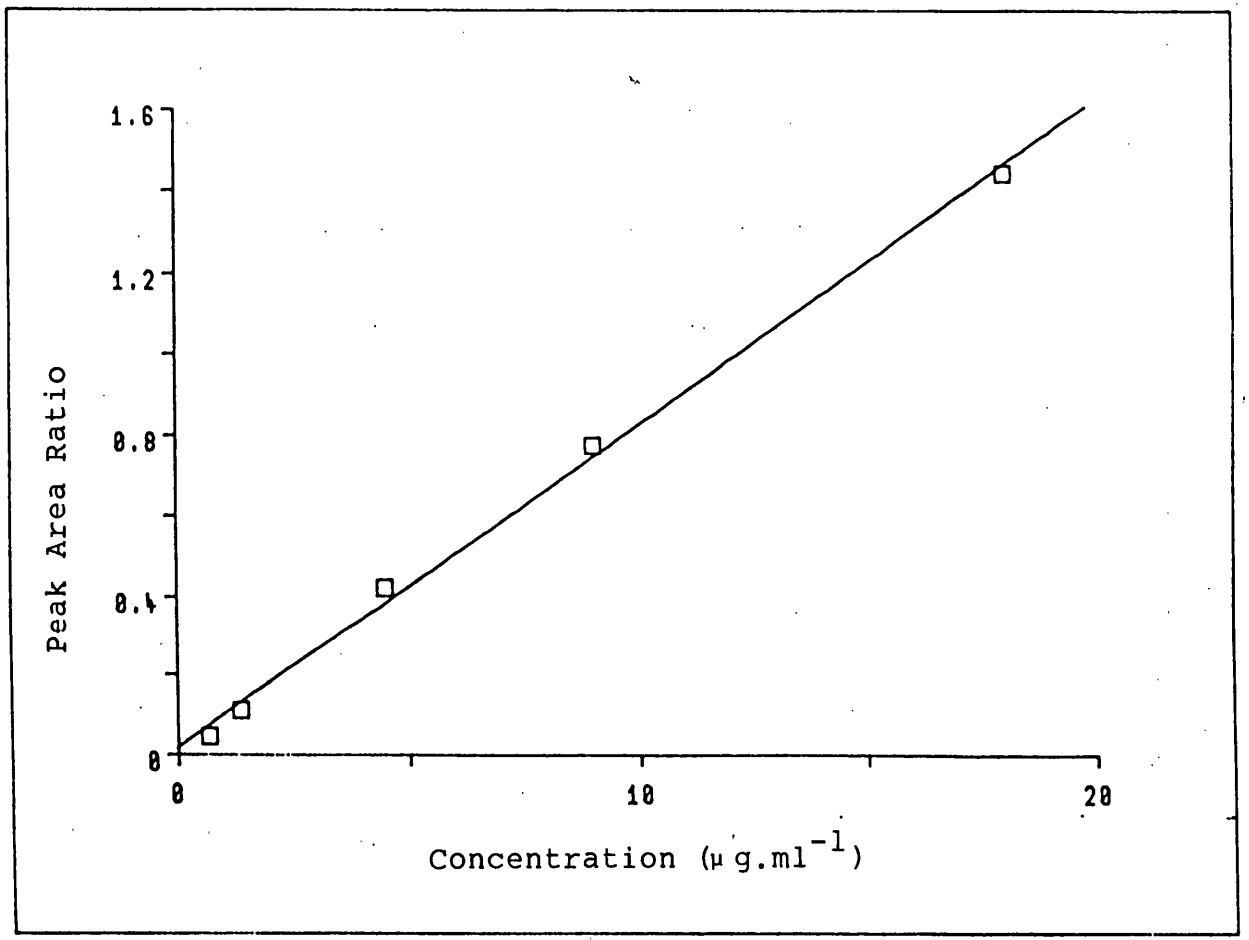


Figure 3.16b Calibration of dipipanone (0.7-18 μ g.ml^{-1}) with norpipanone as internal standard. Conditions as in Fig. 3.15.

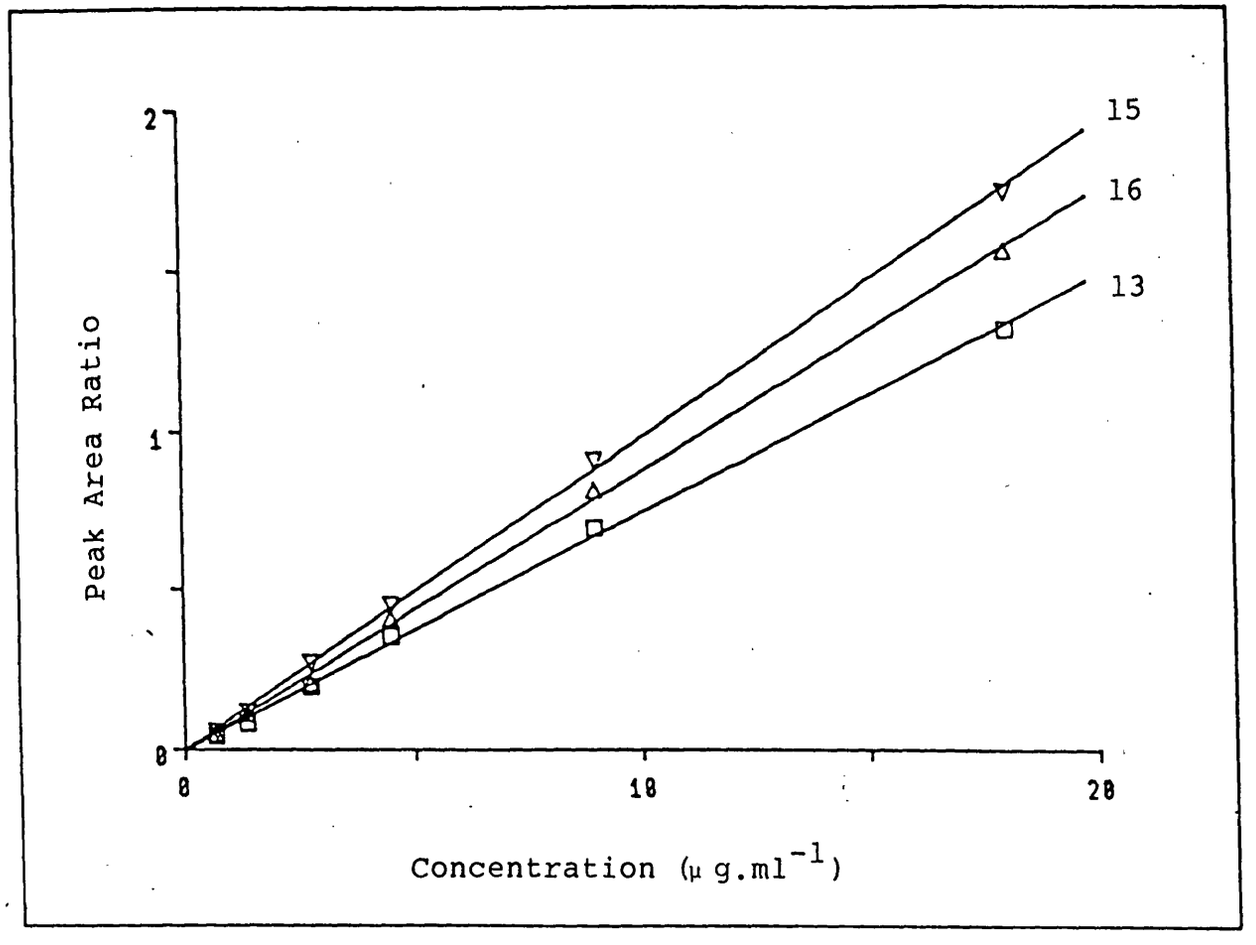


Figure 3.16c Calibration of benzphetamine (13), normethadone (15) and methadone (16), (0.7-18 μg.ml^{-1}) with norpipanone as internal standard. Conditions as in Fig. 3.15.

3.2 Trimethyl silyl-bonded Silica

Hypersil SAS is a 5 μm material with a surface area of about 200 m^2g^{-1} and a mean pore diameter of 12 nm. It consists of an alkyl chain bonded by Si-C linkages to microspherical silica, i.e. it is a C1 material. This material was considered to be suitable for rapid chromatography of the solutes in this work because it has a low carbon loading, 2.6%. The main mechanism of retention of the amines on this stationary phase is influenced by the reversible association of the solute with the hydrocarbonaceous ligand of the stationary phase, and the hydrophobic properties of the solutes.

A 100 x 4.6 mm i.d. column was packed and its performance examined in the reversed phase mode, bearing in mind the possibility of its use with the post-column system. The longer column was chosen to provide more column efficiency (N).

3.2.1 Investigation of Chromatographic Conditions Using Hypersil SAS

Chromatographic parameters were investigated to test the performance of this material with the ultimate aim of getting a short experimental time and good resolution.

3.2.1a Influence of Mobile Phase pH

For Group A compounds it was decided to chromatograph the solutes using the same conditions as with cyanopropyl-bonded silica. It was observed that the compounds were well retained and the retention times were longer than in cyano-bonded silica. Hence the effect of pH was examined. At the chosen pH range (3 - 6.2) it was observed that pH 5.4 would be suitable for Group A compounds whilst Group B compounds required a pH of 3.1. Figures 3.17 and 3.18 show the effect of pH on these two groups of compounds. Reduction in pH shows that the basic amines are forced to protonate more, thus making them more polar and therefore reducing the retention. No selectivity changes were observed as pH was varied.

3.2.1b Influence of Organic Modifiers

An important factor in reversed phase HPLC is the choice of an aqueous-organic solvent mixture that results in selective retention of solutes. It was therefore appropriate to investigate the ratio of methanol-acetonitrile required to separate a mixture of all Groups A and B compounds at their respective pH.

At a buffer pH of 5.4 and a constant 5% v/v acetonitrile, the percentage of methanol-acetonitrile mixture in the mobile phase was increased gradually up to 12.5% v/v each for Group A compounds. Figure 3.19

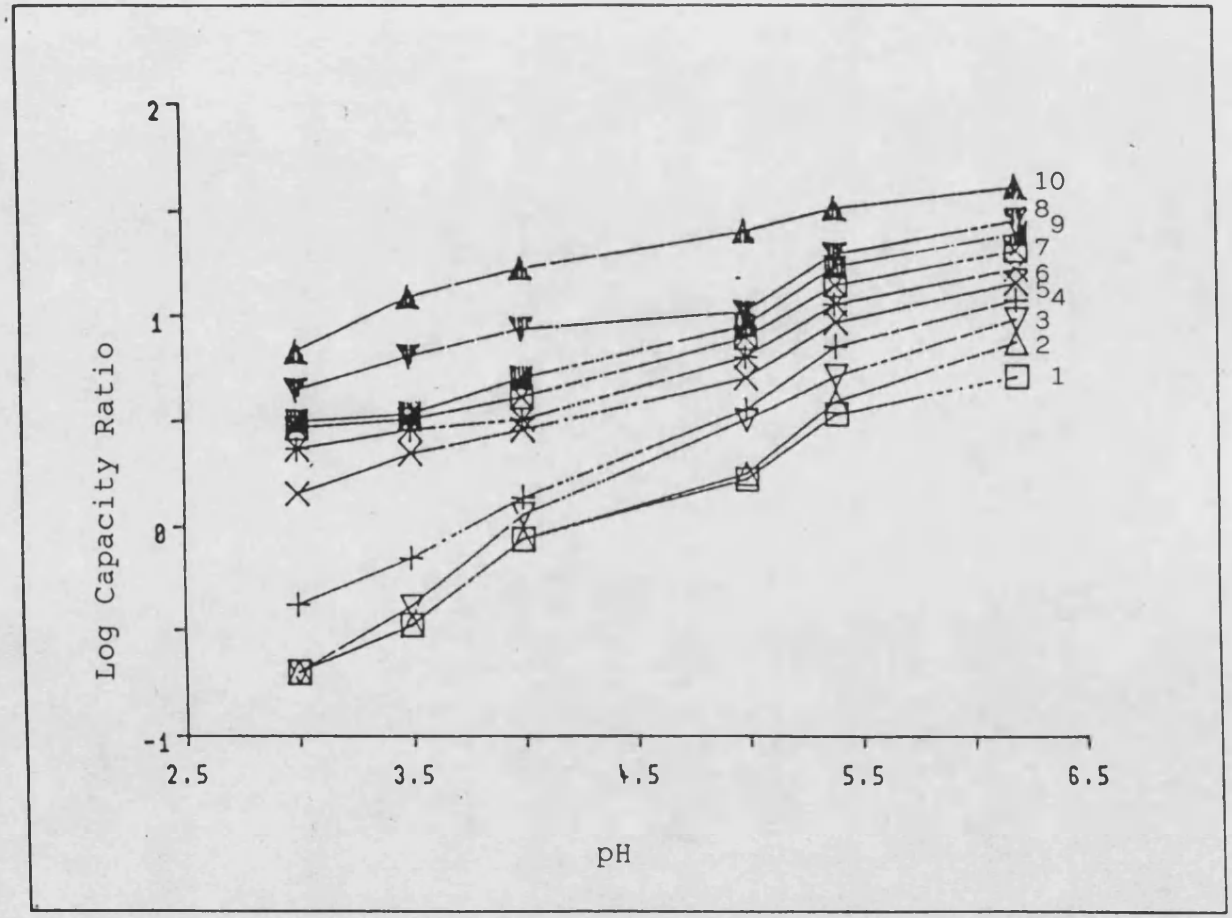


Figure 3.17 Influence of mobile phase pH on Group A compounds. Conditions - Hypersil-SAS (100 x 4.6 mm i.d.). Column, mobile phase was 0.005 M phosphate buffer-methanol-acetonitrile (90:5:5, v/v/v) flow rate was 1 $ml.min^{-1}$ monitored at 205 nm and 0.04 a.u.f.s. Solute code as in Fig. 3.1.

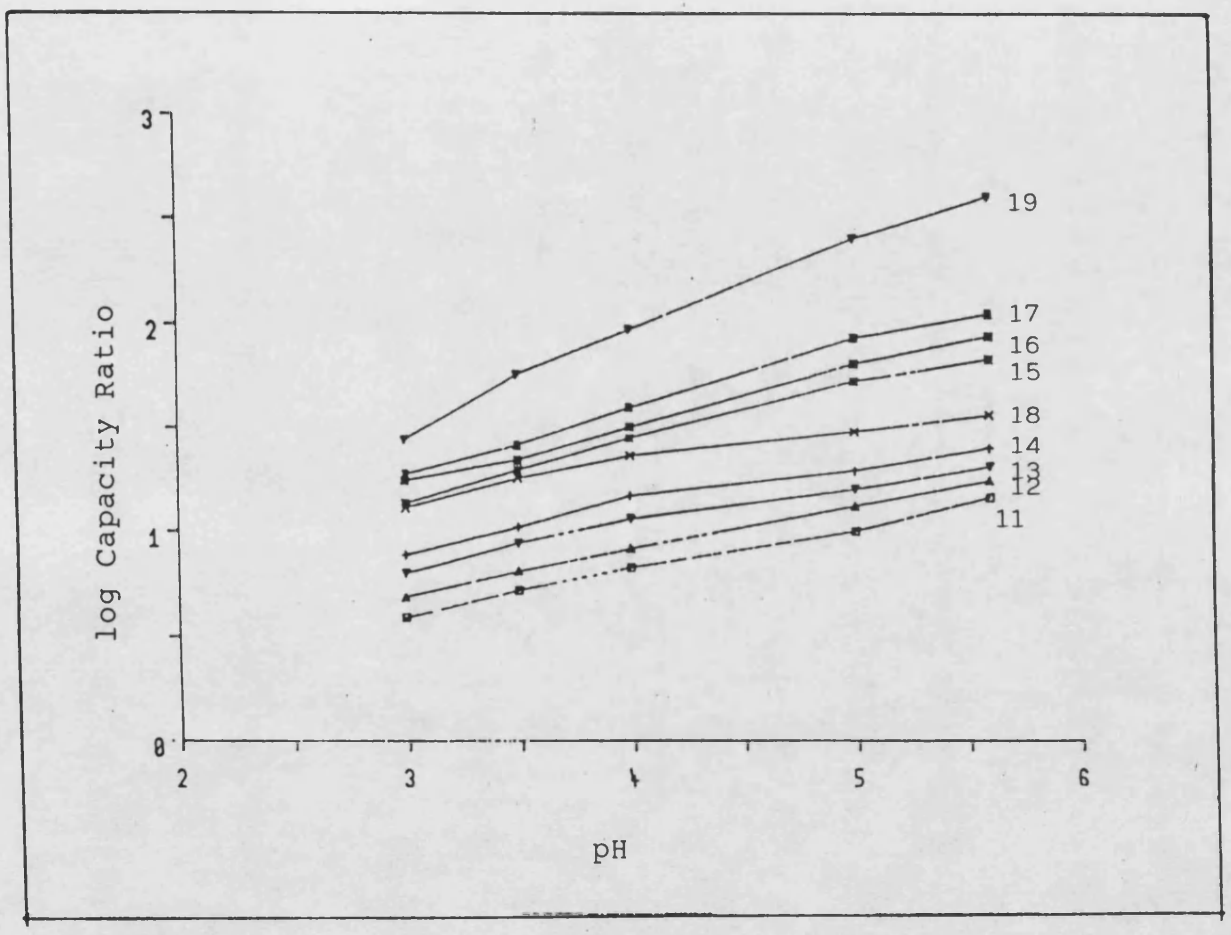


Figure 3.18 Influence of mobile phase pH on Group B compounds, using Hypersil-SAS (100 x 4.6 mm i.d.) column.All other conditions and solute codes as in Figure 3.2.

shows that solute retention decreased almost linearly, although the effect seems to be more on the compounds with methyl groups attached to the nitrogen atom. Group B compounds were also examined, and it was observed that they seem to follow the same pattern as in the cyano-propyl material, Figure 3.20 illustrates this.

3.2.1c Influence of Buffer Concentration

The effect of buffer ionic concentrations on solute retention was examined over the range 0.01 M to 0.1 M, using the respective pH and organic modifier composition pre-determined above. Solute retentions were decreased as buffer concentration was increased, in all cases. Figures 3.21 and 3.22 illustrate this. The range 0.01 M to 0.1 M was chosen as a result of the high retention values obtained as compared to the previous results from the examination of these compounds on cyanopropyl bonded phase.

All the above conditions have been well explained in Section 3.1.2

Figures 3.23 and 3.24 show the chromatograms for Groups A and B respectively under conditions selected for minimum reproducible k' values.

The order of elution of these compounds are basically the same as with the cyano-propyl material except that mephentermine (8) and phendimetrazine (9) had interchanged positions in Group A while in Group B, piritramide (18) was eluted before normethadone (15).

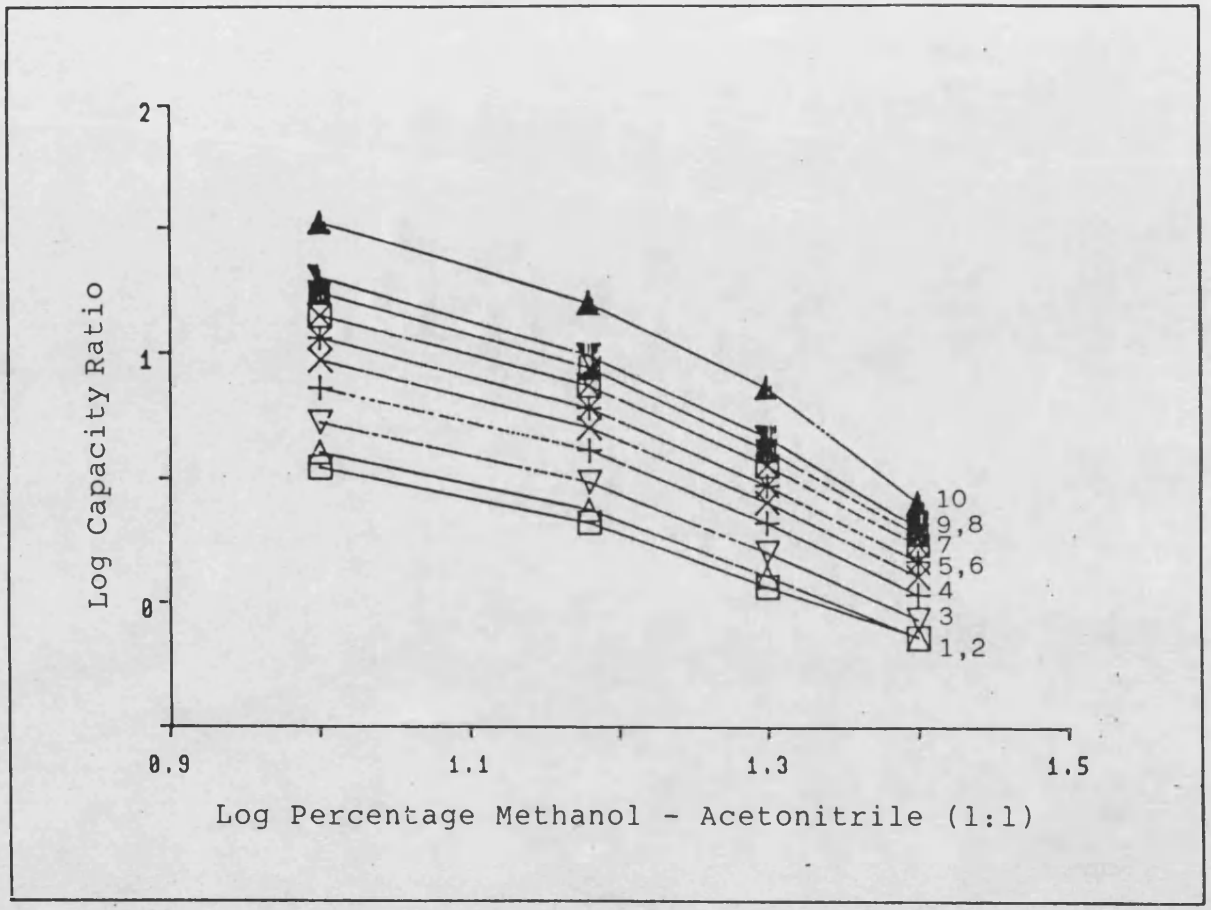


Figure 3.19 Influence of mobile phase methanol-acetonitrile (1:1) content on the retention of group A compounds. Hypersil-SAS (100 x 4.6 mm i.d.) column, mobile phase was 0.005 M phosphate buffer (pH 5.4), flow rate, 1 ml.min^{-1}, monitored at 205 nm and 0.04 a.u.f.s. Solute codes as in Fig. 3.1.

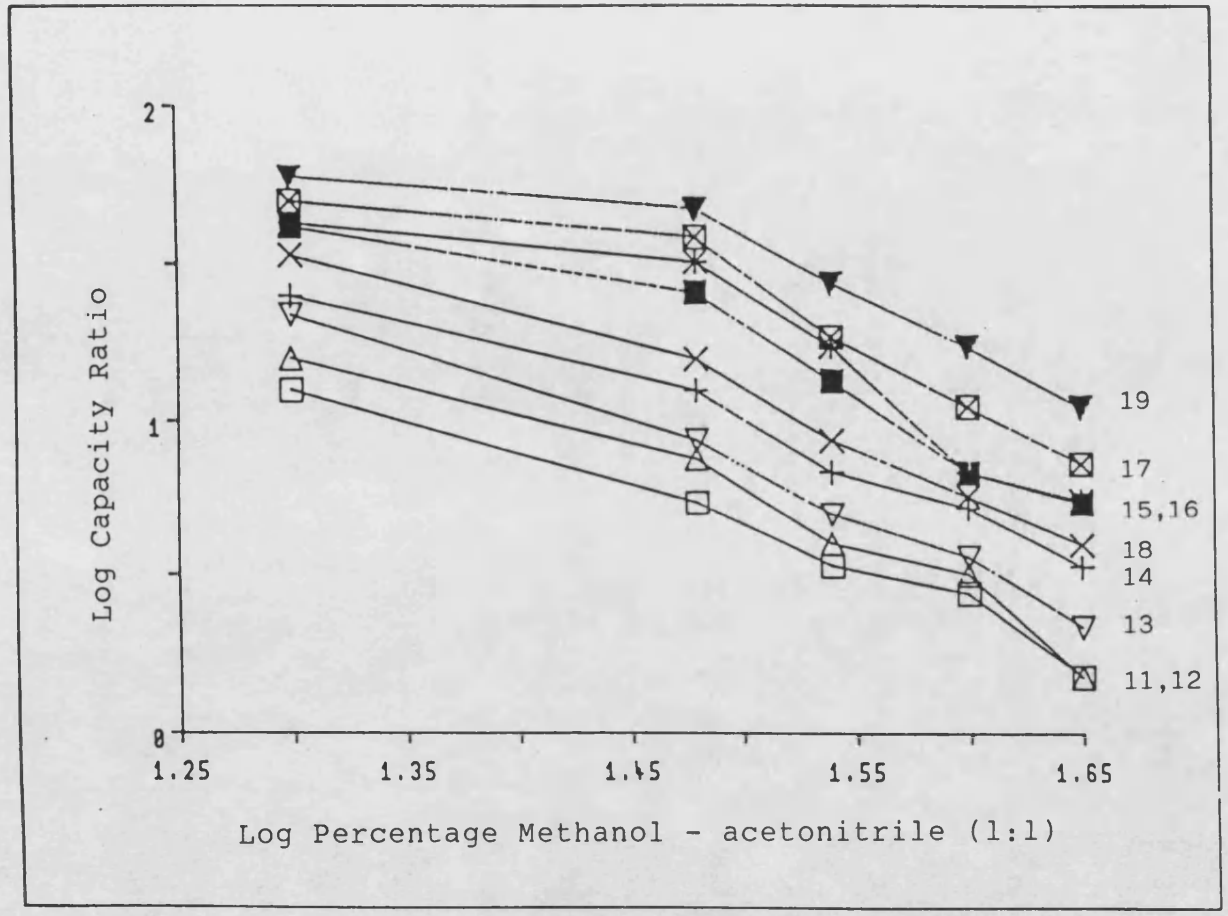


Figure 3.20 Influence of mobile phase methanol-acetonitrile (1:1) content on the retention of Group B compounds. Mobile phase pH 3.1. All other conditions as in Fig. 3.19. Solute codes as in Fig. 3.2.

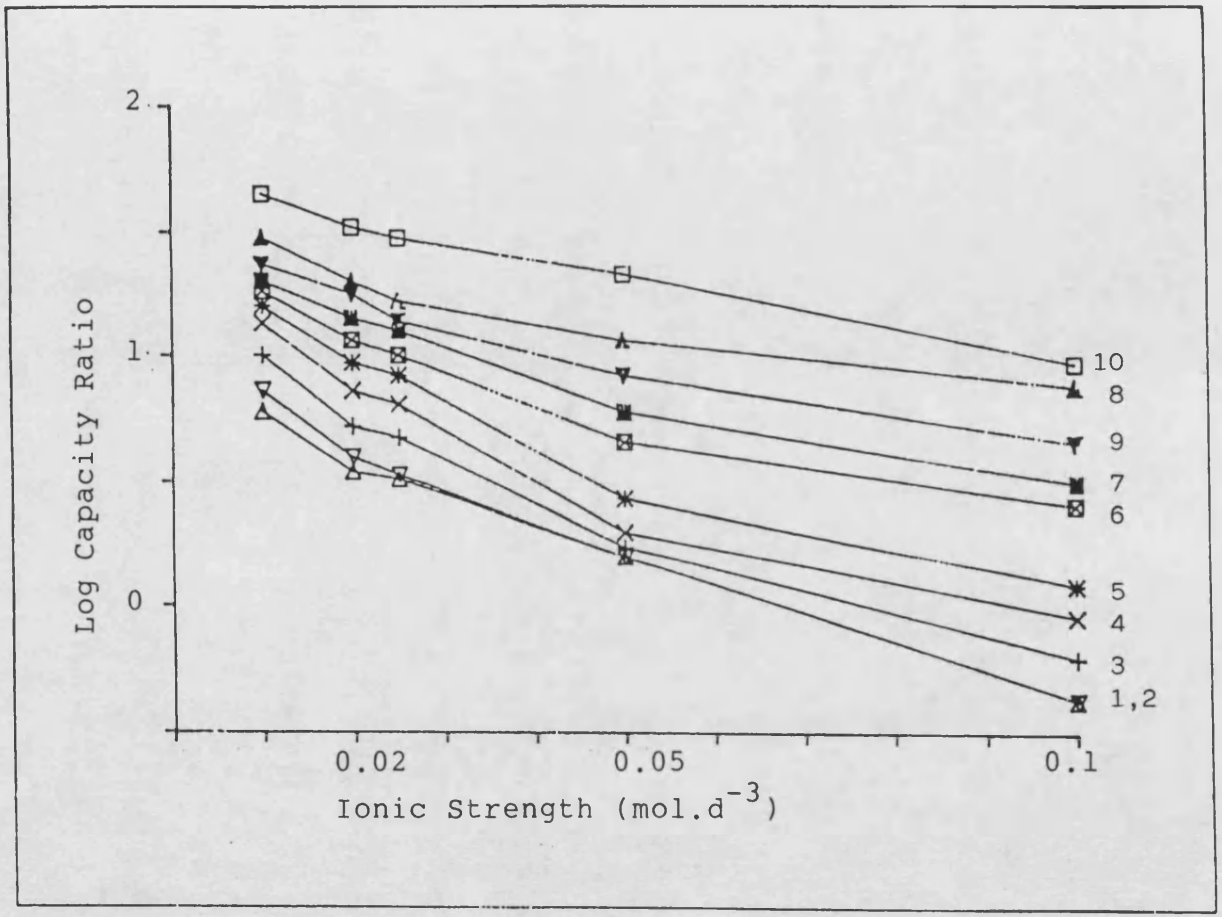


Figure 3.21 Influence of mobile phase buffer ionic strength on the retention of Group A compounds. Condition as in Fig. 3.17, but mobile phase pH was 5.4. Solute code as in Fig. 3.1.

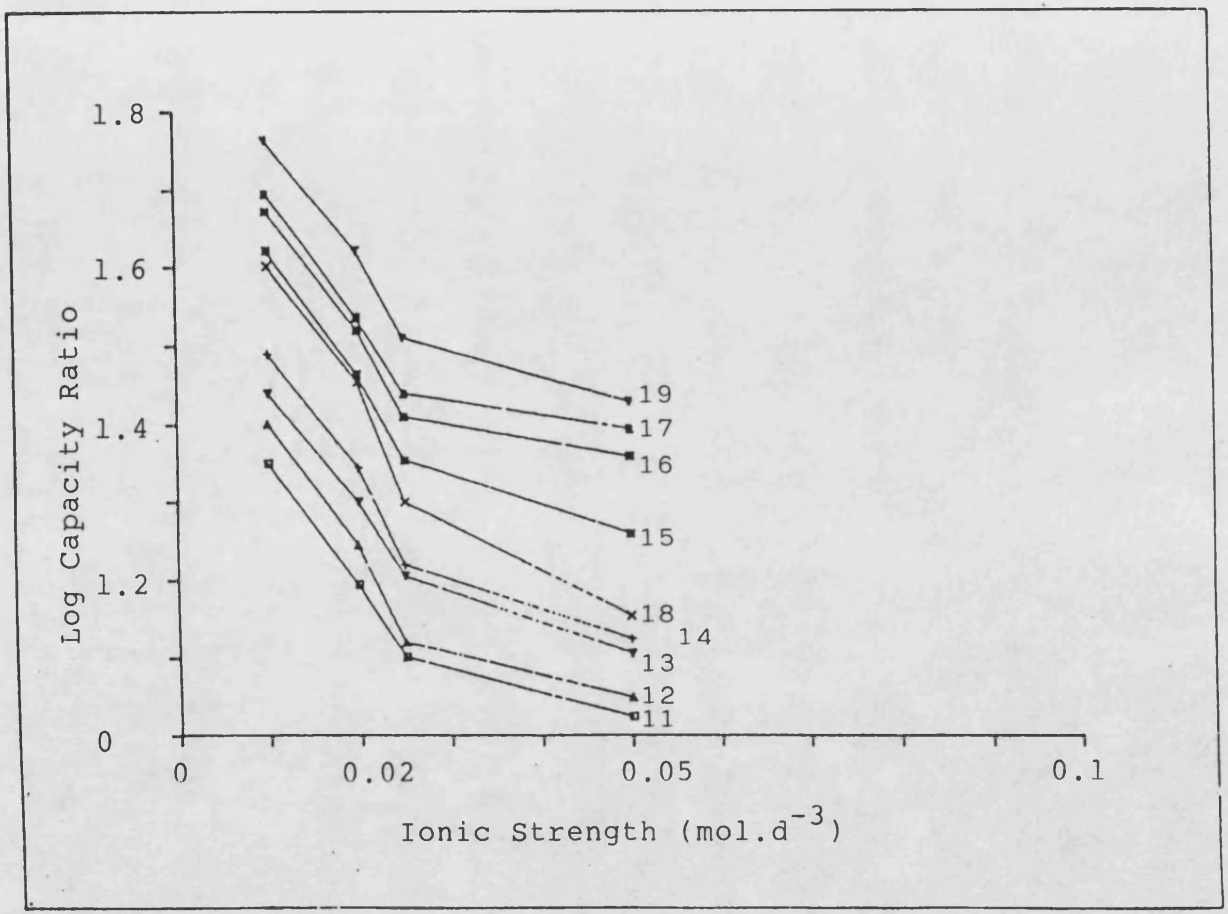


Figure 3.22 Influence of mobile phase buffer ionic strength on the retention of Group B compounds. Conditions as in Figure 3.18 but mobile phase pH was 3.1. Solute code as in Fig. 3.2.

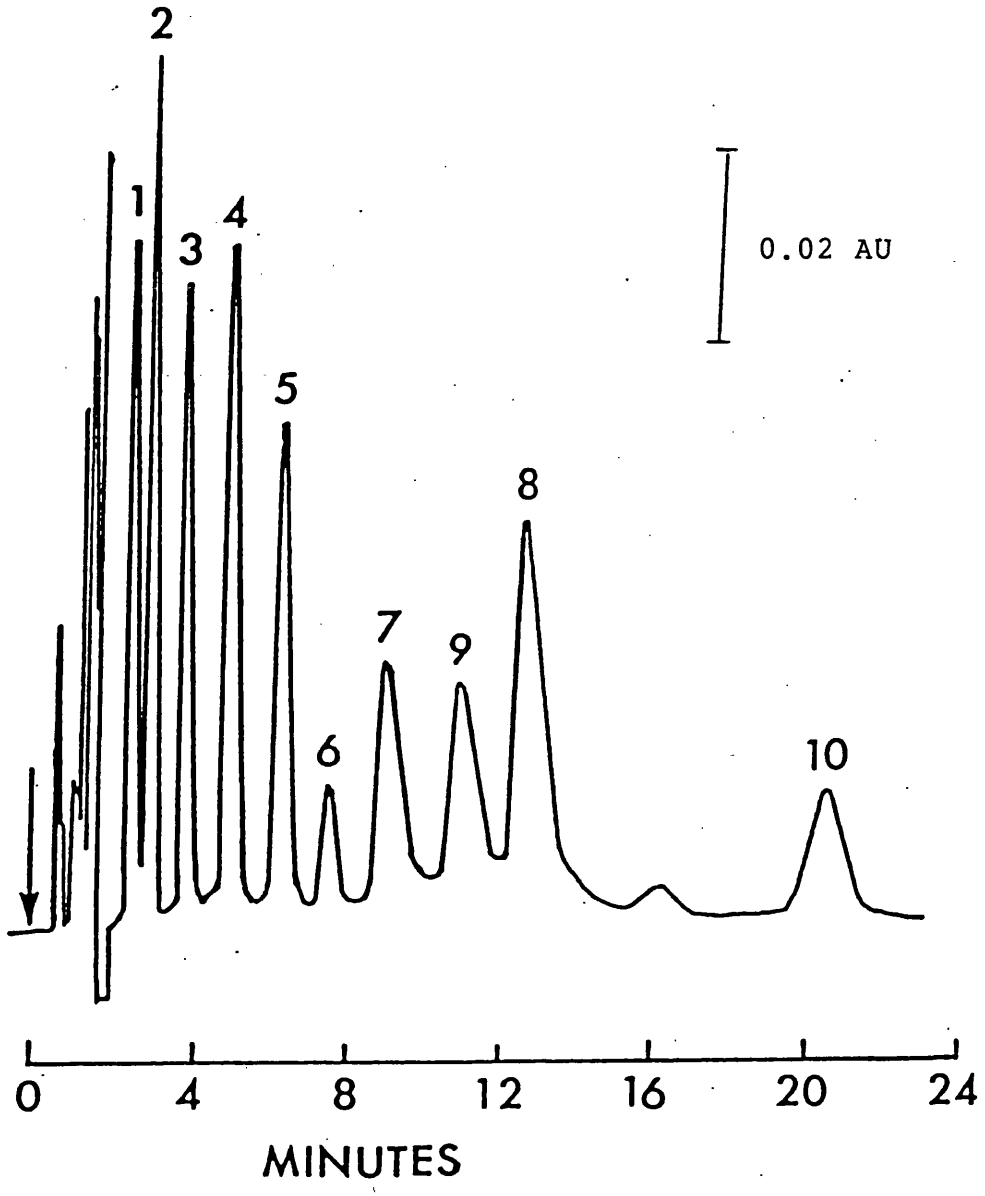


Figure 3.23 Chromatograms of Group A solutes. Conditions: 5 µm Hypersil-SAS (100 x 4.6 mm i.d.) column with 0.02 M phosphate buffer (pH 5.4) - methanol - acetonitrile (90:5:5, v/v/v) at 30°C, 1 ml.min^{-1} flow rate monitored at 205 nM and 0.02 a.u.f.s. Injection volume (20 µl) contained 5-15 ng of solutes. Solute code as in Figure 3.1 .

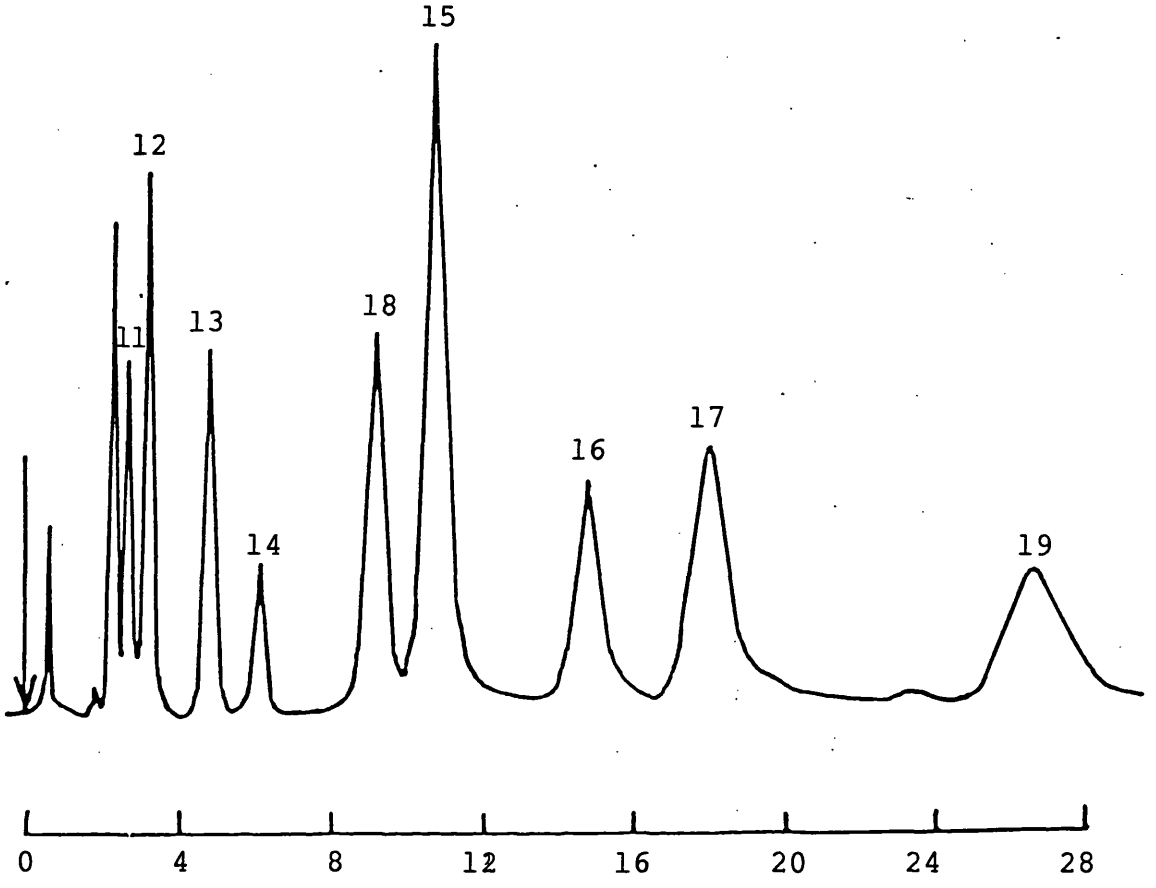


Figure 3.24 Chromatograms of Group B solutes. Conditions: 5 μm Hypersil-SAS (100 x 4.6 i.d.) column with 0.02 M phosphate buffer (pH 3.1)-propan-2-ol-acetonitrile (65: 17.5: 17.5, v/v/v), flow rate 1 $ml.min^{-1}$ monitored at 205 nm. Injection volume (10 μl) contained 10 ng each solute. Solute code as in Fig. 3.18.

These are unexpected, and may well be as a result of the morpholine group in phendimetrazine. The structure of piritramide is different from other compounds in this group and its behaviour is probably greatly influenced by the cyclic nitrogen-atom.

The long retention times obtained for all these compounds on Hypersil SAS was not expected and is probably not caused by interaction with free silanol groups either, because peak shapes (Fig. 3.23-24) were good, with peak asymmetry (As) in the range of 1.0-1.5 (Table 6.1). The long retentions probably reflect the low content of organic modifier and the longer column length employed.

3.2.2 Quantitative Aspect

In view of the fact that good resolutions and selectivities were obtained with the above material under the chosen conditions, the system was then subjected to quantitative investigation with a 100 x 4.6 mm i.d. column using the conditions described in Figure 3.23, for Group A compounds. Five-point calibrations were made for a mixture containing dexamphetamine, methylamphetamine and three metabolites, namely p-hydroxynorephedrine, p-hydroxy-amphetamine and p-hydroxymethylamphetamine. Linear relationships between solute concentrations and peak areas were obtained over the concentration range 10-80 $\mu g.ml^{-1}$ with correlation coefficients in the range 0.998-0.999 and intercepts not significantly different from zero as shown in Table 3.6 and Figures 3.25 and 3.26. Adaptability of the system was tested by packing a 100 mm x 2 mm i.d. column, and using the same conditions as for a 100 mm x 4.6 mm column except reducing the flow rate to 0.5 $ml.min^{-1}$. Similar capacity factors were obtained when tested with Group B compounds. Sensitivity had increased about five times and column efficiency had improved to 3880 in dipipanone (Fig. 3.27) compared to 1520 in the 100 x 4.6 mm i.d. column.

Quantitative investigation was then repeated for some Group A compounds with the Hypersil SAS (100 x 2.1

Figure 3.25 Chromatograms of the highest (a) and lowest (b) concentrations for the calibration of p-hydroxynorephedrine (1), p-hydroxyamphetamine (2), p-hydroxymethylamphetamine (3), dexamphetamine (5), methylamphetamine (7). Injection volume (10 l) contained 10-80 $\mu g.ml^{-1}$. Conditions as in Fig. 3.23.

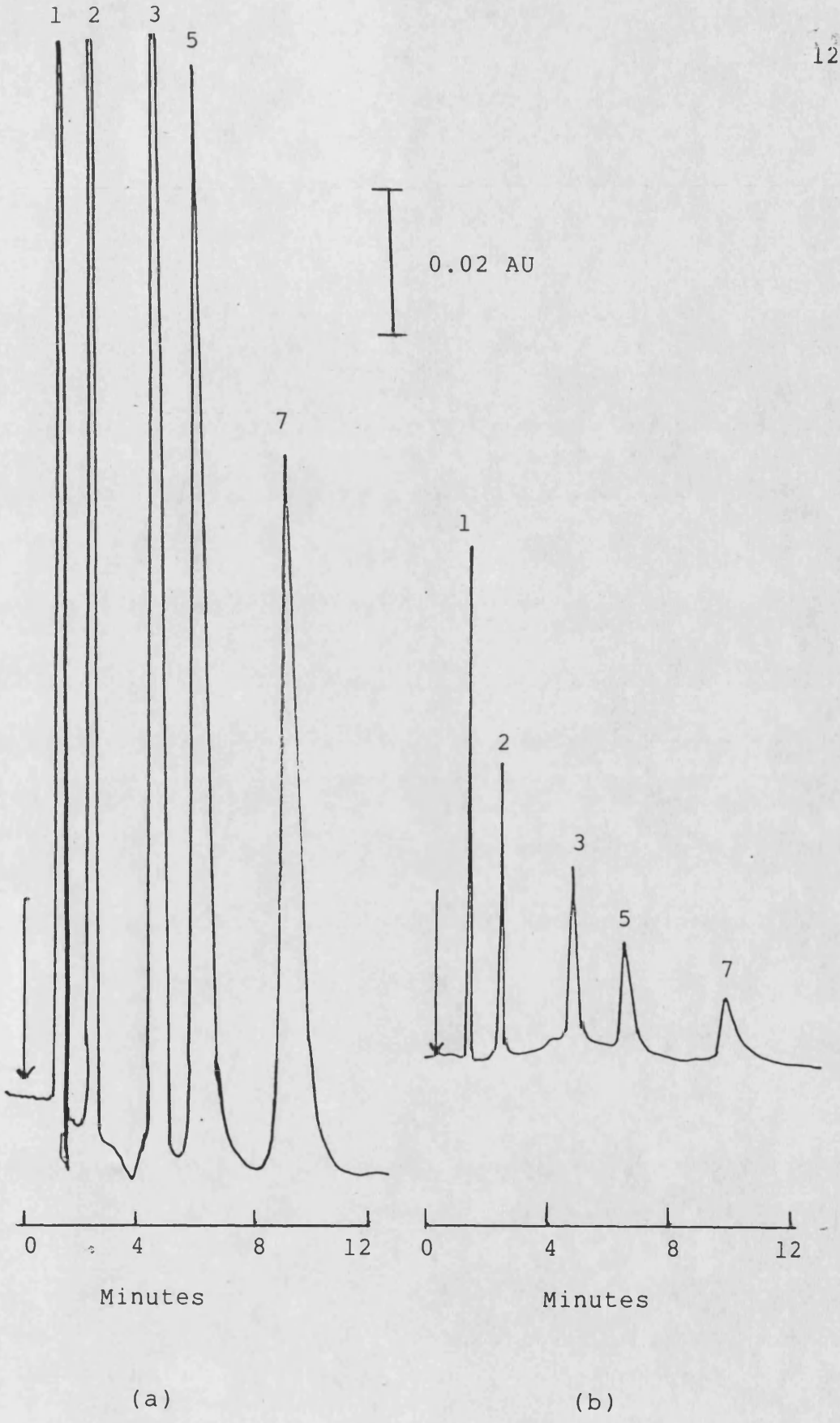


Figure 3.25. Code as on opposite page

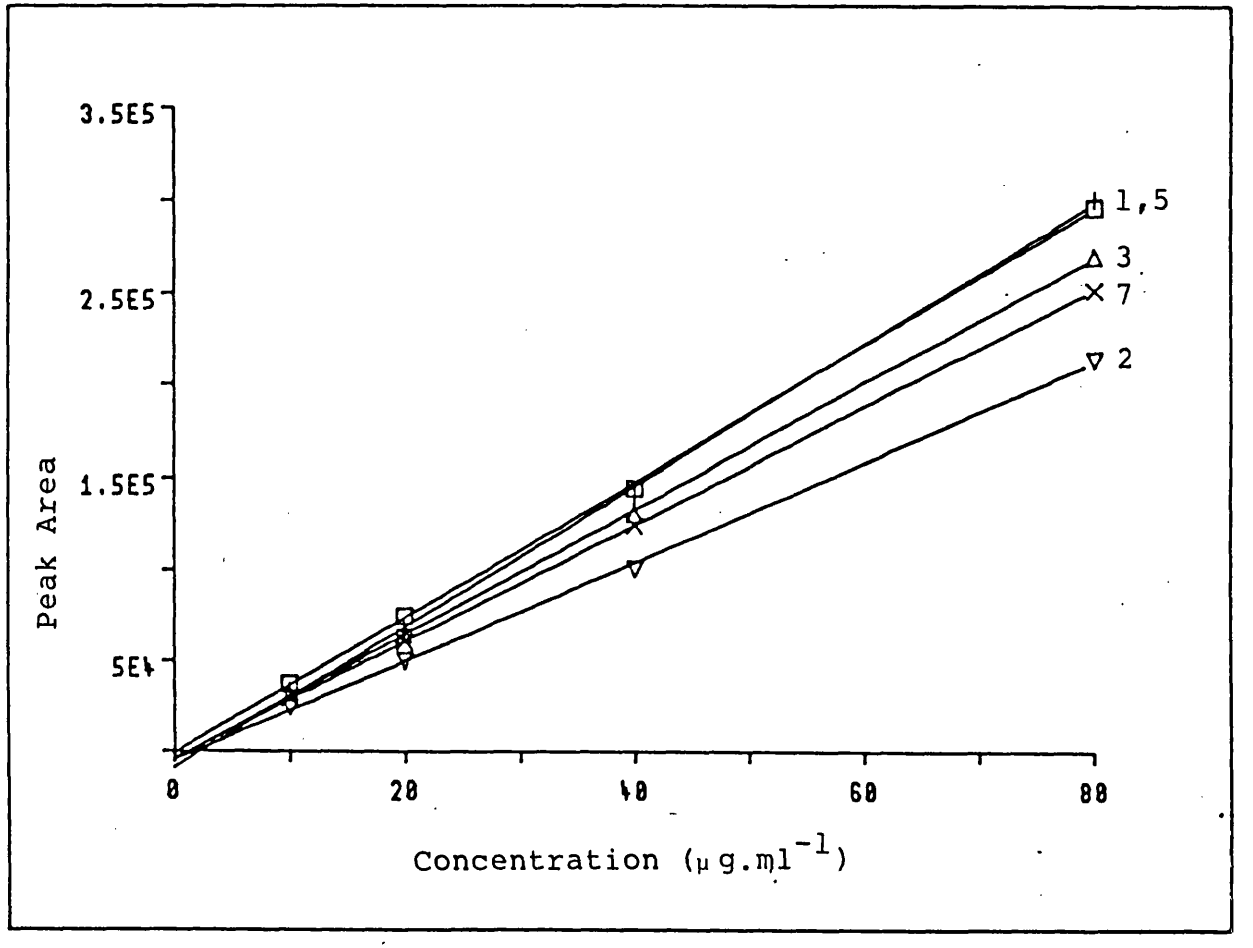


Figure 3.26 Calibrations of some Group A compounds. Conditions and code as in Figure 3.25.

Table 3.6. Statistical analysis of the calibration of some Group A compounds (10-80 $\mu g.ml^{-1}$). p-hydroxynorephedrine (1), p-hydroxyamphetamine (2), p-hydroxymethylamphetamine (3), dexamphetamine (5), methylamphetamine (7). Conditions as in Fig. 3.25.

	1	2	3	5	7
Correlation coefficient	0.9997	0.9991	0.9997	0.9990	0.9999
Slope	3700.7	2709.3	3415.2	3844.2	3191.9
Std. dev. of slope	43.921	56.055	38.879	85.287	3.3379
Intercept	-539.04	-4594.7	-4070	-8581	-3517.3
Std. dev. of intercept	2024.7	2584	179.2	3931.5	153.87
RSD of slope (±, %)	0.03	0.04	1.14	2.22	0.11

mm i.d.) column in a concentration range of 2-20 $\mu g.ml^{-1}$ using a Spectra Physis SP8100 liquid chromatograph with autosampler, oven and 20 µl sample loop, SP8440 UV-Vis detector and SP4200 computing integrator. Peak areas were used for the calibration here. Linear relationship was also obtained (Figure 3.29) and correlation coefficients were between 0.991 and 0.997 with small negative intercepts. Figure 3.28 shows the highest and lowest concentrations for calibration.

Calibration of Group B compounds using the conditions in Figure 3.27 was also carried out over the concentration range of 1-4 $\mu g.ml^{-1}$ for all the compounds. A linear relationship was obtained with correlation coefficients ranging from 0.995-0.999, Table 3.8 shows the statistical analysis while Figures 3.30(a) and 3.30(b) are the highest and lowest concentrations for calibration.

The results obtained are reproducible, both resolution and column efficiencies are acceptable. It shows this is a sensitive and versatile system and it is very simple to operate. The only drawback could be the column life at pH 3.1 which is used for Group B compounds, and the very short alkyl chain which renders the stationary phase to be stripped easily.

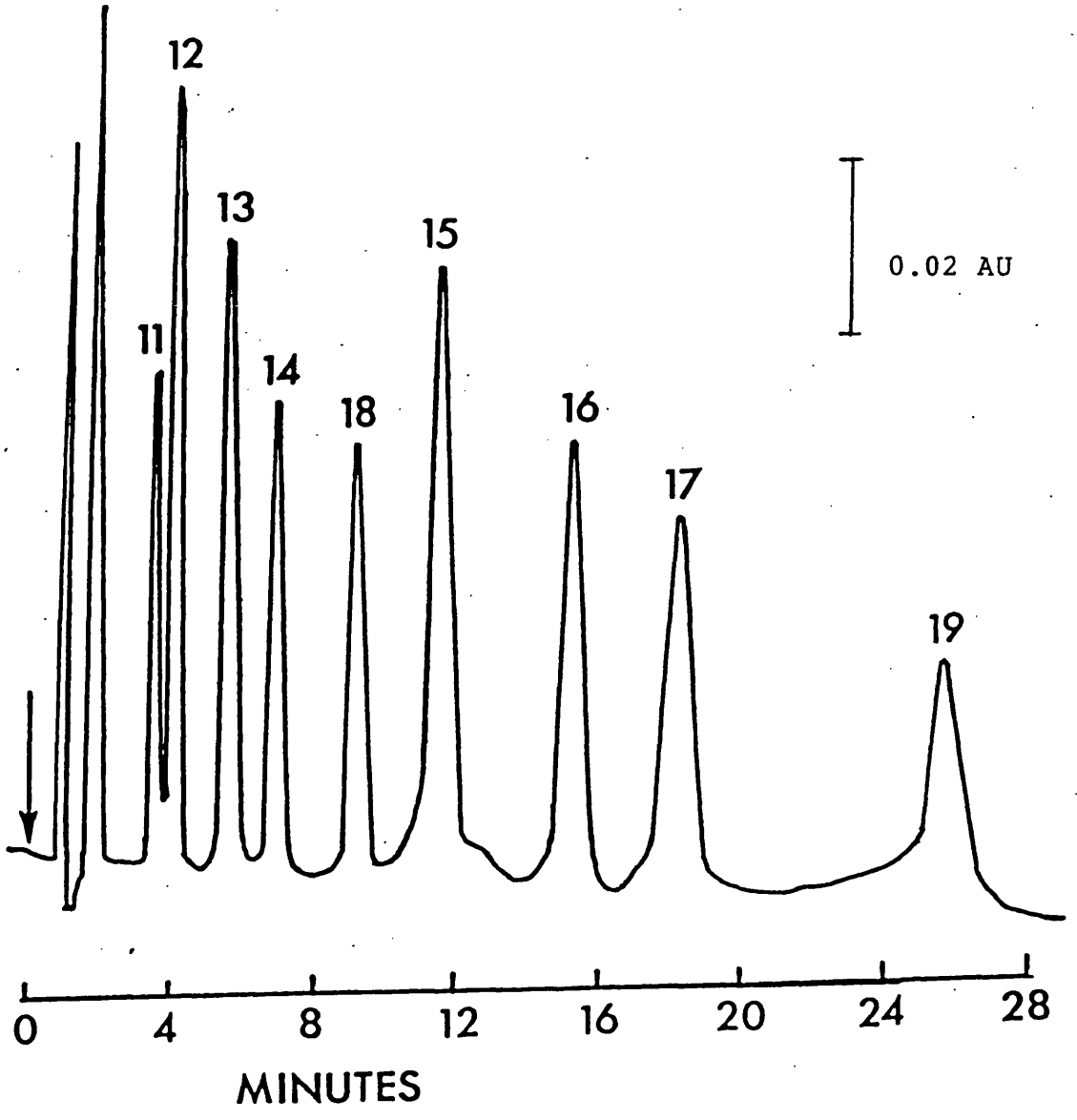


Figure 3.27. Typical chromatograms of Group B compounds.
5 µm Hypersil-SAS (100 x 2.1 mm) column, flow rate 0.5 ml.min^{-1} contained about 3 ng each solute. All other conditions as in Figure 3.24.

Figure 3.28 Chromatograms of the highest (a) and lowest (b) concentration for the calibration of p-hydroxynorephedrine (1), p-hydroxyamphetamine (2), p-hydroxymethylamphetamine (3), dexamphetamine 95) and methyl-amphetamine (7) (2-20 $\mu g.ml^{-1}$). Conditions as in Figure 3.23 (except for flow rate 0.5 ml/min).

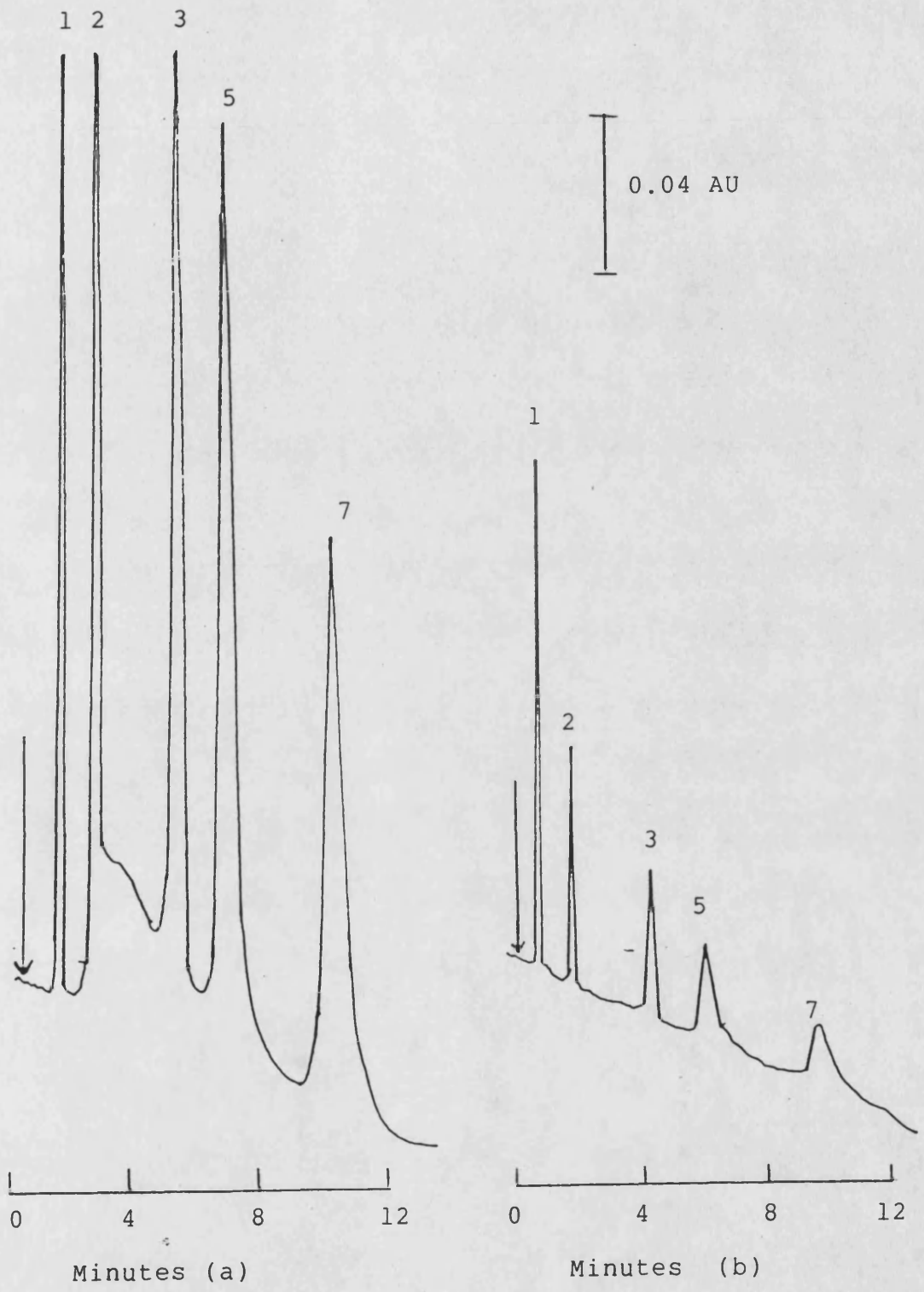


Figure 3.28. Codes as on opposite page

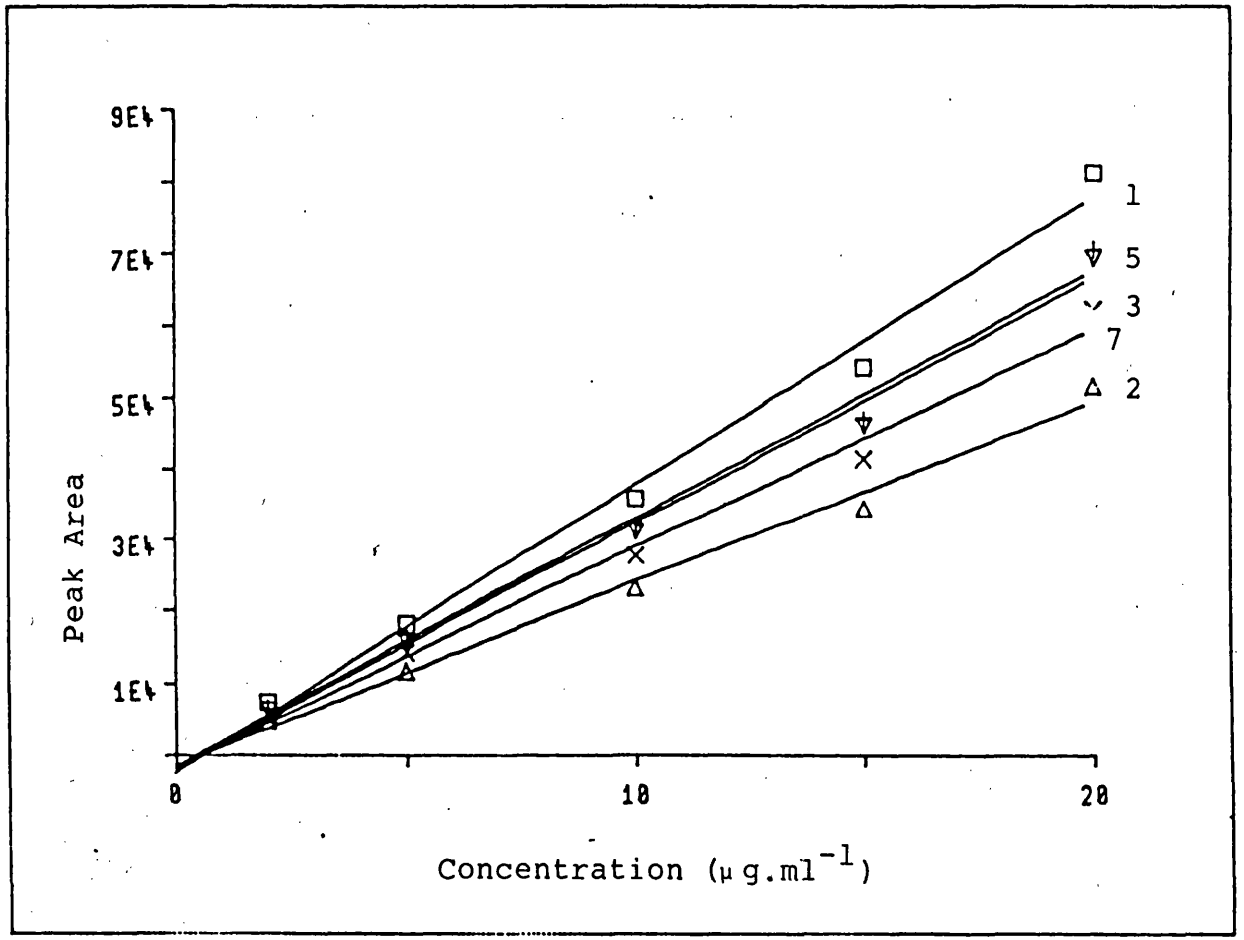


Figure 3.29 Calibration of some Group A compounds. Conditions and solutes as in Figure 3.28.

Table 3.7. Statistical analysis of the calibration of some Group A compounds (2-10 $\mu g.ml^{-1}$). p-hydorxynorephedrine (1), p-hydroxyamphetamine (2), p-hydroxymethylamphetamine (3), dexamphetamine (5), methylamphetamine (7). Conditions as in Figure 3.28.

	1	2	3	5	7
Correlation coefficient	0.9910	0.9911	0.9915	0.9911	0.9913
Slope	4017.9	2544.8	3427.2	3457.1	3065.5
Std. dev. of slope	221.33	138.98	183.11	189.5	166.06
Intercept	-2338.5	-1303.2	-1848.9	-1393.1	-1638.8
Std. dev. of intercept	2718	1706.7	2248.6	2327.1	2039.2
RSD of slope (±, %)	5.51	5.4	5.34	5.4	5.4

Figure 3.30(a) Chromatogram of the highest concentration (4.0 $\mu g.ml^{-1}$) for the calibration of Group B compounds. Conditions and solute code as in Figure 3.27.

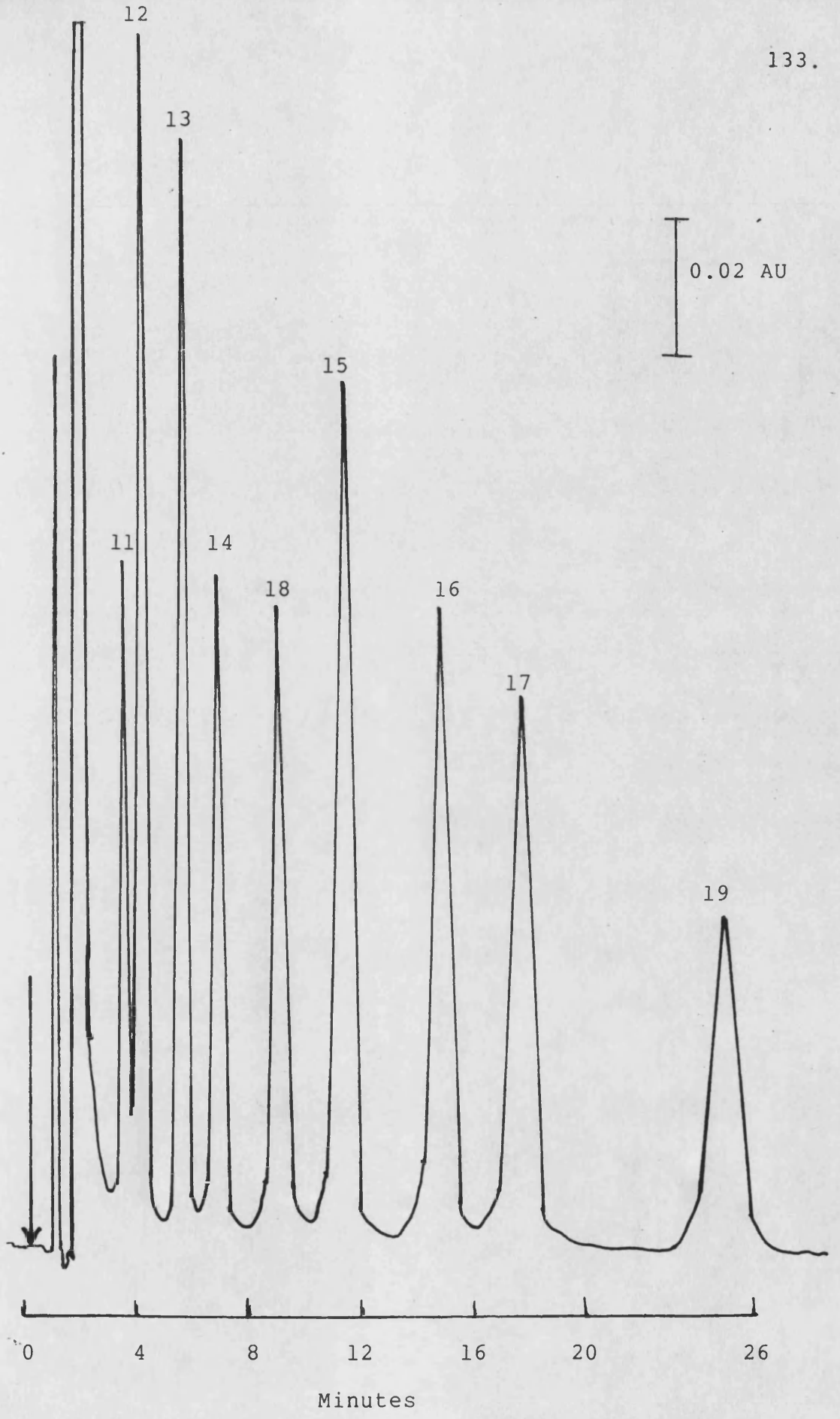


Figure 3.30(a)

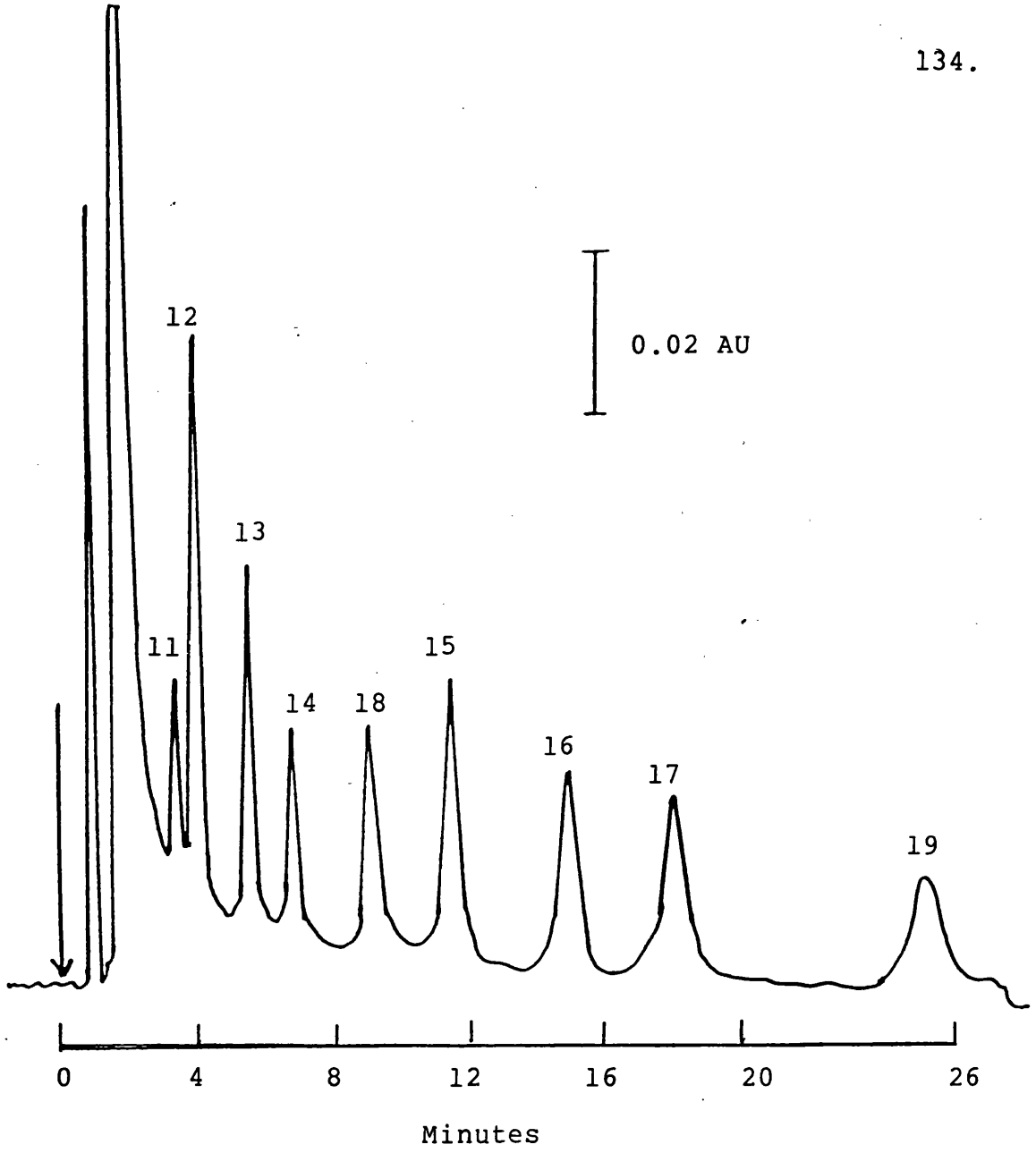


Figure 3.30(b) Chromatogram of the lowest concentration (1.0 $\mu g.ml^{-1}$) for the calibration of Group B compounds. Conditions and solute code as in Figure 3.27.

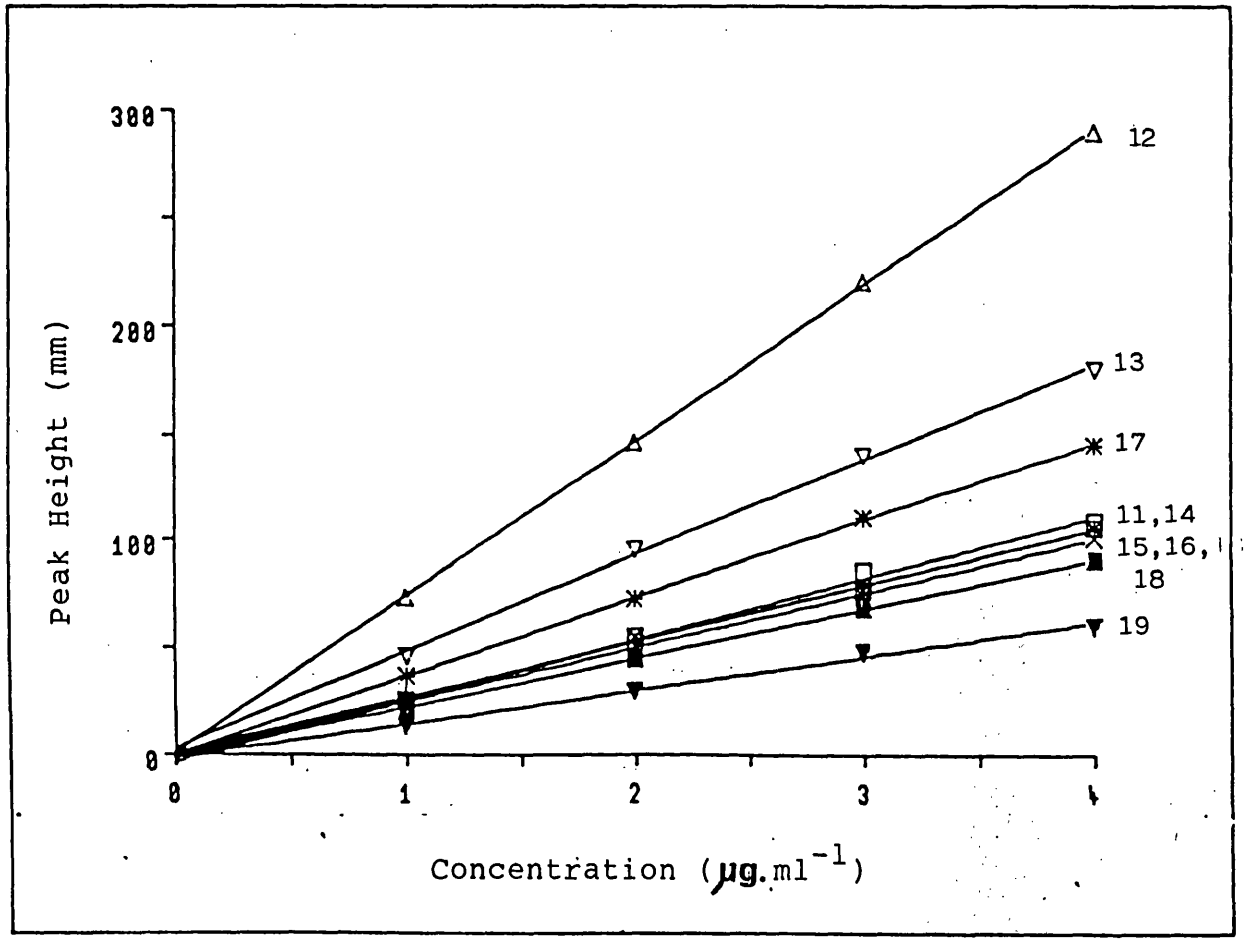


Figure 3.31 Calibrations of Group B compounds. Conditions and code as in Figure 3.30.

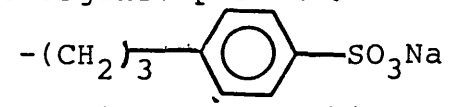
Table 3.8. Statistical analysis of the calibration of Group B compounds. Conditions and solute code as in Figure 3.30.

	11	12	13	14	18	15	16	17	19
Correlation coefficient	0.9958	0.999	0.998	0.9993	0.9998	0.9997	0.9962	0.9969	0.994
Slope	28.2	72.5	44.7	25.7	24.9	36.2	25.8	22.7	15.6
Std. dev. of slope	1.2961	0.592	1.4248	0.48	0.265	0.42	1.13	0.17	0.85
Intercept	-3.0	1.0	3.5	1.50	-0.02	0.5	1.0	-0.5	-1.0
Std. dev. of intercept	3.5496	1.62	3.902	1.31	0.725	1.162	3.1	0.47	2.32
RSD of slope (±, %)	4.59	0.82	3.19	1.87	1.06	1.16	4.38	0.75	5.45

3.3 Ion-Exchange Bonded Silica

3.3.1 Nucleosil SA

Nucleosil SA is a silica-based cation exchanger that is strongly acidic. It is a spherical, totally porous material, stable between pH range 1-9. It has a constant permeability, even when the strength of the mobile phase ions vary and or when the pH value of the eluent is altered. The functional group is sulphonic acid, and the organic part is:



The pore diameter of this material is 10 nm, the pore volume is 1.0 $ml.g^{-1}$ with a specific surface area of 350 $m^2.g^{-1}$ and ion-exchange capacity of 1 $mequiv.g^{-1}$ and particle size 5.0 ± 1.5 μm.

It is generally known that for ion exchangers, different ions show different degrees of affinity to the ion exchange sites, for example the order of increasing affinity for a cation exchanger is as follows:-

$$H^+ < Na^+ < NH_4^+ < C_s^+ < Ag^+$$

Divalent ions are more attracted to the ion exchange group than monovalent ions and trivalents more than divalents. The greater the strength and effective radius of the ions, the stronger the attraction to the ion exchange groups.

The primary process in cation exchange is adsorption-desorption of charged basic solutes with a

permanently charged anionic stationary phase. There are secondary mode effects inherent in ion exchangers, which are mainly adsorptive in nature. The suitability of this material to chromatograph compounds in Groups A and B was investigated. A 100 x 4.6 mm i.d. column was initially packed and tested, but due to very high retention values obtained, a 50 x 4.6 mm i.d. column was finally used for this work.

3.3.1a Influence of Mobile Phase pH

The study of the influence of pH on the retention of Group A compounds was studied using 0.05 M phosphate buffer-methanol-acetonitrile (90:5:5, v/v/v). Retention of all solutes were reduced as pH was increased, but large capacity ratios were obtained even at a pH of 7.2.

A change in the pH value of the mobile phase will only alter the equilibrium between the sample molecules and the functional ionic groups of the stationary phase. When the pH of the system is decreased, the protonated amines are formed more readily:

$$RNH_2 + H^+ \rightleftharpoons R\overset{+}{N}H_3,$$

and therefore competition of the charged amine ions for the cation exchange sites are increased and subsequently retention is increased. It has been

suggested that in cation exchange an increase in pH is equivalent to an increase in solvent strength, therefore a decrease in retention would be expected.

3.3.1b Influence of Organic Modifiers

The effect of increasing the quantity of organic modifier is similar to what was observed in the reversed phase system for cyanopropyl and Hypersil SAS columns. As solvent strength is increased retention was reduced almost linearly for these compounds.

3.3.1c Influence of Buffer Ionic Strength

The ionic strength of the mobile phase is proportional to the concentration of the ions in solution. Increasing the ionic strength at a constant pH caused a reduction in retention, this is because there is more competition for ion-exchange sites between the protonated solute and buffer ions. Therefore retention was reduced. There were some selectivity changes, but in general they were small and not an important consideration.

It was decided to use 0.2 M KH_2PO_4 (pH 5.0)-methanol-acetonitrile (82.5: 10: 7.5, v/v/v) at 57°C with Group A compounds. This is because this condition gave a good resolution between the compounds and analysis time was short. This condition gave good peak shapes that are comparable with the

cyanopropyl column. Figures 3.32 and 3.33 show typical chromatograms of some Group A and Group B compounds, respectively.

3.3.2 Quantitative Aspect

The above condition was used for the calibration of some solutes in Group A over the concentration range of 0.25-1.0 $\mu g.ml^{-1}$. Linear relationships were obtained with correlation coefficients 0.999, and slope RSD ± 5, Table 3.9).

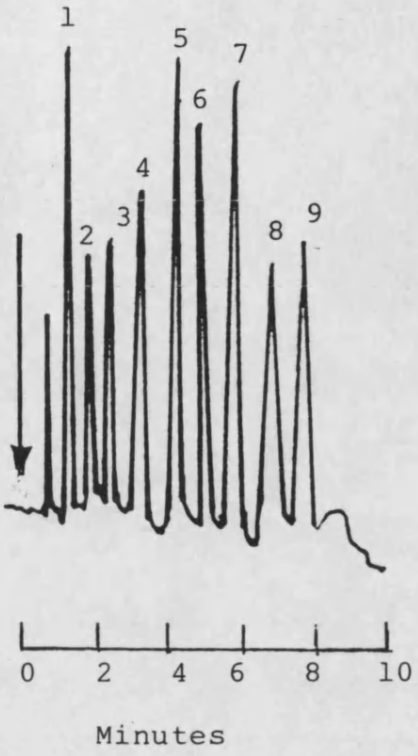


Figure 3.32 Chromatograms of the separation of some Group A compounds.

Conditions

Stationary phase	Nucleosil SA 5 µm (50x4.6 mm id.)
Mobile phase	0.2 M KH_2PO_4 (pH 5.0)- methanol-acetonitrile (82.5:10:7.5 v/v/v)
Temperature	57°C
Flow rate	1.0 ml.min^{-1}
Injection volume	10 µl
Detection	0.02 a.u.f.s. at 205 nm.

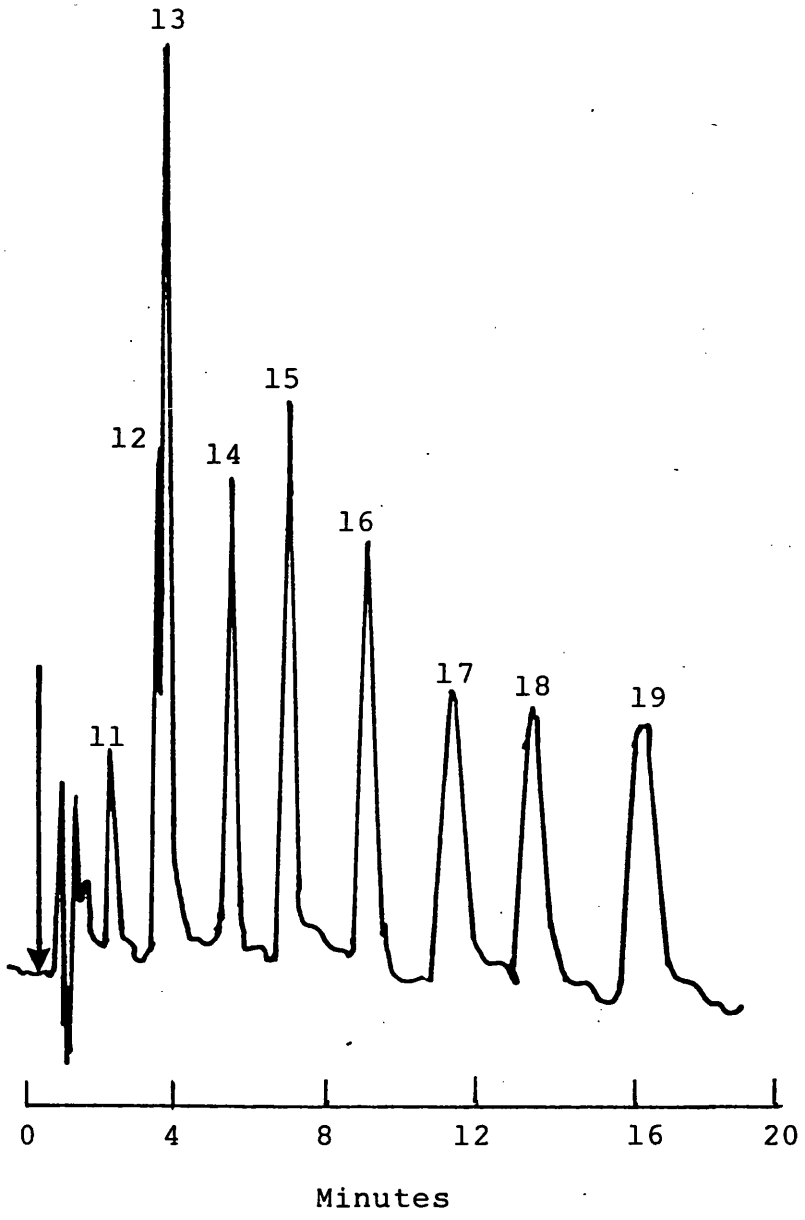


Figure 3.33. Chromatograms of the separation of Group B compounds. Legends as in Figure 3.2.

Conditions

Stationary phase	Nucleosil SA 5 μm (50 x 4.6 mm id.)
Mobile phase	0.2 M KH_2PO_4 (pH 5.0) - Propan-2-ol - acetonitrile (60:20:20 v/v/v)
Temperature	57° C
Flow rate	1.0 ml.min^{-1}
Injection volume	10 μl
Detection	0.02 a.u.f.s. at 205 nm

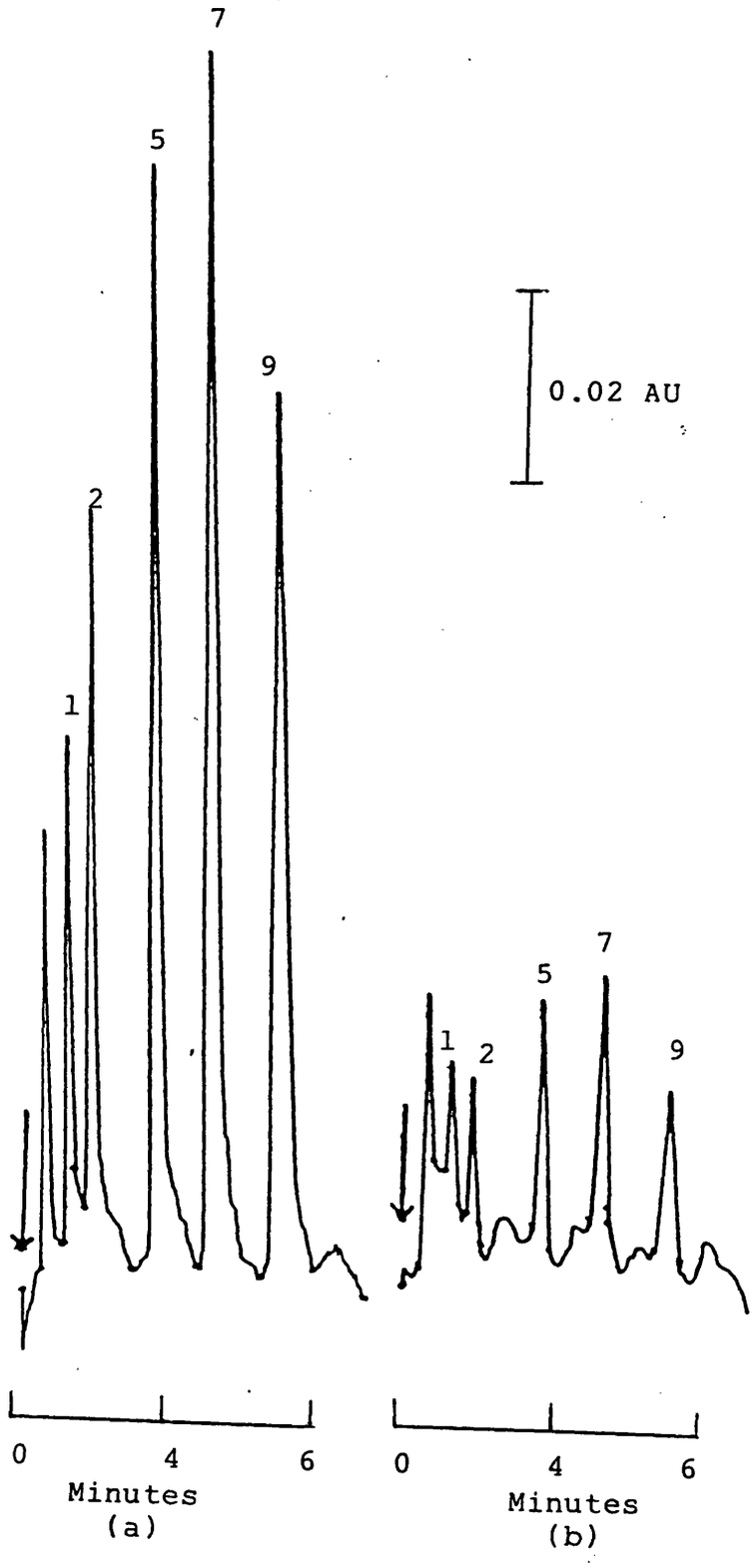


Figure 3.34. Chromatograms of the highest (a) and lowest (b) concentrations for the calibration of some Group A compounds. Conditions and solute code as in Figure 3.32.

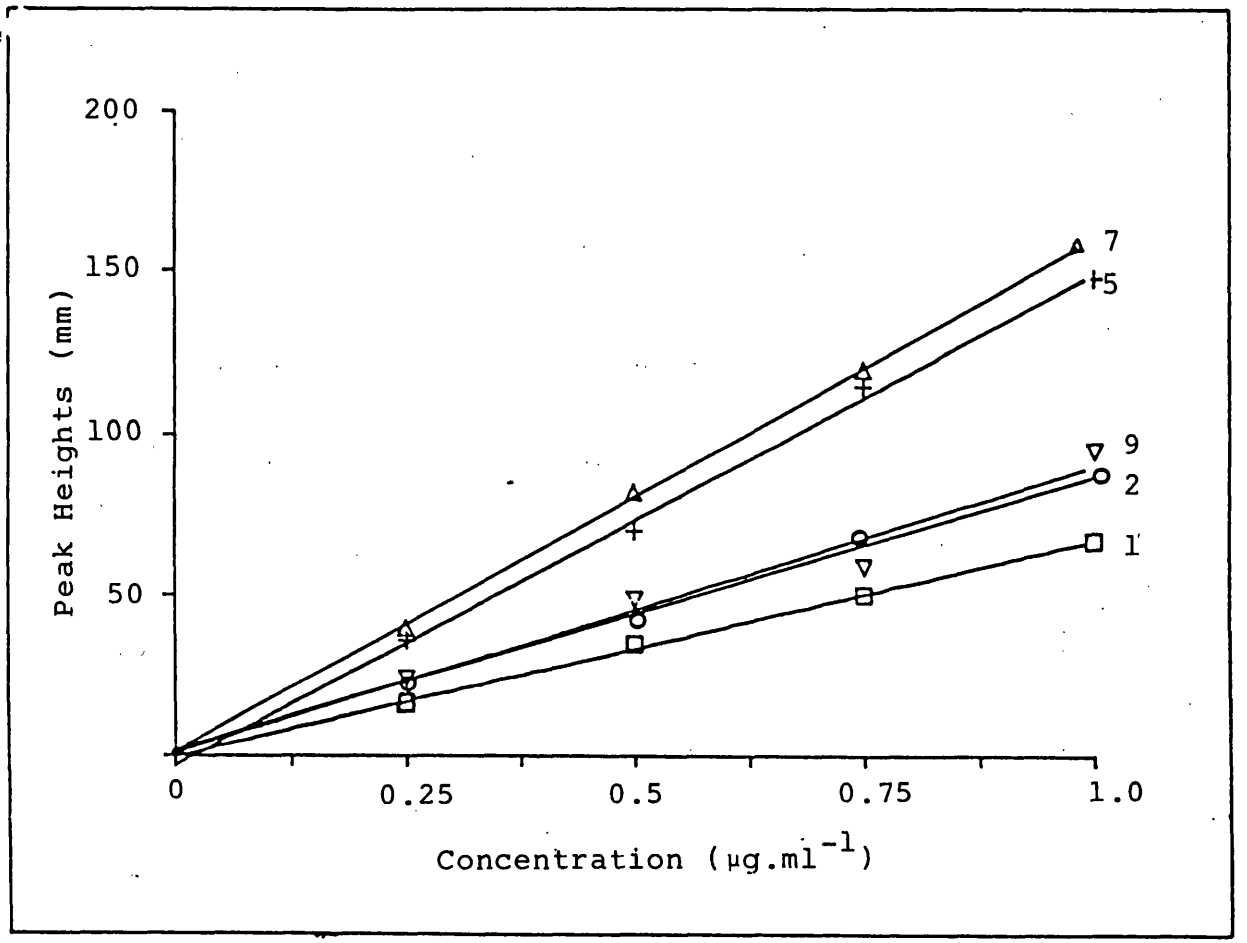


Figure 3.35. Calibration of some Group A compounds. Conditions as in Figure 3.32. Solute codes as in Figure 3.1.

Table 3.9. Statistical analysis of the calibration of some Group A compounds, p-hydroxynorephedrine (1), p-hydroxyamphetamine (2), dexamphetamine (5), methylamphetamine (7) and mephentermine (9). (0.25-1.0 $\mu g.ml^{-1}$). Conditions as in Figure 3.32.

	1	2	5	7	9
Correlation coefficient	0.9980	0.9921	0.9963	0.9995	0.9941
Slope	16.8	22.3	38.1	40.7	31.1
Std. dev. of slope	0.529	0.959	1.634	0.656	1.024
Intercept	0.0002	1.0	-3.0	-0.5	-6.0
Std. dev. of intercept	1.4491	9.259	4.475	1.80	4.64
RSD of slope (±, %)	3.15	4.48	4.29	1.61	3.29

3.4 Other Chromatographic Materials Investigated

3.4.1a PRP-1

This is a 10 µm porous polystyrene divinylbenzene polymer adsorbent material with physical and chemical properties very similar to those proposed for XAD-2, but PRP-1 has a higher surface area and is suitable for HPLC applications. Its major advantage is that it is stable over the pH range 1.5 to 13. Lee et al. (52) demonstrated the versatility of this material by using it to separate nucleosides and their corresponding bases at both low and high pH, but it was discovered that those bases had very short retention and separation efficiency.

A 150 x 4.1 mm commercial column with PRP-1 material was tried with the same conditions used for Spherisorb-CN, Hypersil-SAS and Nucleosil SA, but the peak shapes were very broad with long retention times. Increasing organic modifier content to 36% (0.025 M citrate-phosphate buffer, pH 4, -propan-2-ol-acetonitrile, 54; 18; 18% v/v/v) did not improve peak shapes adequately. The addition of 1% dichloromethane to the mobile phase has been shown (53) to double column efficiency when mainly aqueous mobile phases are used, however this did not improve column efficiency with the above mobile phase.

3.4.1b Hypersil-Phenyl

This is a 5 µm particle marketed principally as a reversed phase material for the separation of moderately

polar materials. The carbon loading of this material is about double that of trimethylsilyl-bonded silica (5.0%), with phenyl as the functionality. This material would be more hydrophobic than the cyano-phase under reversed-phase conditions but less retentive than C_8 materials. It was selected because it was expected to be more stable than Hypersil SAS which has a short alkyl chain (C_1) and therefore the silyl bonding is more exposed to attack by mobile phase.

A 100 x 2.1 mm i.d. column was packed as described in Section 2 and tested under the same conditions as Hypersil SAS, using Group A compounds. Peak shapes were poor and asymmetric even when organic modifier and buffer concentrations were increased. Work with this material was therefore discontinued.

The problem of peak tailing and excessive retention when basic compounds are chromatographed on octadecyl-silica materials has been overcome by the addition of amines, such as N,N-dimethylamino-octane to the mobile phase (54). This type of compound masks the acidic silanol groups responsible for the problem and is very effective. Unfortunately this approach is not compatible with post-column ion-pair detection.

SECTION 4

DETERMINATION OF DISSOCIATION CONSTANTS (pKA) AND PARTITION COEFFICIENTS (Log P)

SECTION 4. AN EXAMINATION OF THE EFFECTS OF THE MOBILE PHASE COMPOSITIONS USED IN SECTION 3 UPON SOLUTE DISSOCIATION CONSTANT (pKa) AND PARTITION COEFFICIENT (log P)

4.1 Examination of the Dissociation Constants (pKa) of the compounds investigated

The basic nature of the drugs of abuse examined in this thesis is of interest, because it is difficult to define a simple physico-chemical reference state for such solutes for which dynamic (RP-HPLC) and static (e.g. octan-1-ol-water distribution, aqueous solubility) parameters are comparable (42). Furthermore, various experimental procedures such as spectrophotometry, potentiometry and conductometry have been employed to generate pKa values.

The Bronsted-Lowry theory is the most useful and widely accepted description of the ionization of both acids and bases. The underlying concept of this theory is the definition of a base as a substance which can accept a hydrogen ion. The pKa of a compound is usually expressed as the negative logarithm (-log) of its acid dissociation constant. The pKa value is said to be a convenient numerical way to compare the relative acidity or basicity of ionising compounds in aqueous or miscible solvent-aqueous solutions (56, 57).

The earlier investigation (Section 3) of the effect of mobile phase pH upon solute capacity ratio (Section 3.1.2a) showed that for all the solutes examined, capacity ratios continued to fall at pH values 0.6 to 2.8 pH units below literature (pKa - 2) values. It was anticipated that minimum k' values would be obtained, but even at a pH of 4.0 minimum k' values had not been reached for solutes whose literature pKa values ranged from 6.6 to 10.4. It was therefore decided to examine the effect that the mobile phase had upon the determination of pKa values and to compare these with literature values.

4.1.1 EXPERIMENTAL PROCEDURES

Potentiometric titration was used according to the procedure described by Albert and Serjeant (58), and is described in Section 2.6. The performance of the equipment and procedure was tested using sodium benzoate as recommended by Albert and Serjeant and the results obtained are compared in Table 4.1 with those given by ref. 58.

The following equation was used to calculate the pKa values of the bases determined in this work:

$$pKa = pH + \log [BH^+] - \log [B] \qquad ...4.1a$$

Table 4.1. The determination of the pKa of sodium benzoate at 20°C

0.1 M HCl (ml)	pH	pKa (Expt)	pKa (Lit)
0	6.40	-	-
0.5	5.05	4.10	4.14
1.0	4.70	4.10	4.15
1.5	4.50	4.13	4.16
2.0	4.30	4.12	4.16
2.5	4.18	4.08	4.15
3.0	3.95	4.11	4.15
3.5	3.80	4.14	4.16
4.0	3.58	4.12	4.16
4.5	3.30	4.08	4.15
5.0	3.10	-	-

The average pKa from this experiment is 4.11 ± 0.03 which is comparable to the literature value of 4.15 ± 0.01. It was therefore considered that the apparatus and procedure was sufficiently accurate for pKa determinations.

The dissociation of a weak base may be taken as:-

$$BH^+ + H_2O \rightleftharpoons H_3O^+ + B \quad \ldots 4.1b$$

$$K_a = \frac{[a_{H_3O^+}][a_B]}{[a_{BH^+}]} \qquad \ldots 4.2$$

where, K_a = thermodynamic dissociation constant

$a_{H_3O^+}$ = thermodynamic activity of hydrated proton

a_B = thermodynamic activity of undissociated base

a_{BH^+} = thermodynamic activity of cation.

The thermodynamic activities are used in order to allow for the departure from ideality of real solutions, and the thermodynamic dissociation constant is independent of the concentration. In all determinations in this study, activity corrections were made where necessary. The pKa values for nineteen compounds were determined using eight different solvent compositions chosen to examine the effect of HPLC mobile phase composition and are given in Table 4.2.

4.1.2 RESULTS OF pKa DETERMINATIONS IN VARIOUS SOLVENT COMPOSITIONS

The pKa values for all the solutes studied in Section 3 were determined under the conditions given in Table 4.2 and the results are presented in Tables 4.3 to 4.4.

Table 4.2. Experimental conditions for pKa determinations

1.	Ethanol-Water (50; 50% v/v) at 20°C
2.	Ethanol-Water (50; 50% v/v) at 40°C
3.	Ethanol-0.02 M KCl solution (50; 50% v/v) at 20°C
4.	0.02 M KCl solution at 20°C
5.	0.02 M KCl solution at 40°C
6.	0.02 M KCl solution - Organic modifier* (90; 10% v/v) (Group A Compounds) at 20°C
7.	" " " at 40°C
8.	0.02 M KCl solution - Organic modifier** (65; 35% v/v) (Group B compounds) at 20°C
9.	" " " at 40°C
10.	Phosphate buffer (pH 5.4) - Organic modifier* (90; 10% v/v) (Group A compounds) at 30°C
11.	Phosphate buffer (pH 4.1) - Organic modifier** (65; 35% v/v) (Group B compounds) at 40°C

* Acetonitrile-Methanol (50; 50% v/v)

** Acetonitrile-Propan-2-ol (50; 50% v/v).

Table 4.3. Determination of pKa of Group A Compounds

COMPOUNDS	pKa[1,56,57] EtOH-H_2O at 20°C Lit Values	pKa found according to conditions in Table 4.2							
		1	2	3	4	5	6	7	10
p-OH-Norephedrine		9.29±0.02	8.18±0.01	7.62±0.02	9.25±0.05	8.51±0.04	9.34±0.01	9.18±0.03	8.80±0.04
p-OH-Amphetamine	9.30	9.32±0.05	8.22±0.03	7.91±0.03	9.30±0.01	8.62±0.01	9.41±0.04	9.21±0.03	8.85±0.02
p-OH-N-Methylamphetamine		9.55±0.01	8.41±0.03	8.22±0.05	9.45±0.02	8.80±0.03	9.80±0.01	9.28±0.02	9.01±0.02
2-Phenylethylamine	9.83[57]	9.72±0.01	8.57±0.04	8.35±0.01	9.61±0.04	9.01±0.02	9.85±0.05	9.36±0.01	9.12±0.01
Dexamphetamine	9.90	9.88±0.02	8.72±0.01	8.41±0.03	9.82±0.01	9.25±0.01	9.96±0.01	9.41±0.01	9.25±0.03
Methylamphetamine	10.10	10.05±0.02	9.01±0.05	8.62±0.1	9.96±0.06	9.42±0.03	10.21±0.02	9.70±0.01	9.4±0.01
Phenmetrazine	8.40	8.41±0.03	7.50±0.02	6.90±0.03	8.43±0.01	7.71±0.04	8.54±0.01	7.86±0.04	7.20±0.05
Phendimetrazine	7.60	7.65±0.05	6.71±0.03	6.25±0.01	7.70±0.02	6.95±0.01	7.91±0.03	7.33±0.02	6.52±0.03
Mephentermine	10.40	10.51±0.03	9.32±0.08	8.95±0.02	10.48±0.01	9.73±0.01	10.48±0.04	9.75±0.01	9.81±0.03
Chlorphentermine	9.60	9.74±0.02	8.64±0.04	8.10±0.01	9.70±0.05	8.97±0.04	10.15±0.03	9.39±0.01	10.02±0.01

Table 4.4. Determination of pKa of Group B Compounds

COMPOUNDS	$pKa^{(1,56,57)}$ EtOH-H_2O at 20°C[2] Lit Values	pKa found according to conditions in Table 4.2							
		1	2	3	4	5	8	9	11
Pethidine HCl	8.70	8.40±0.01	6.90±0.01	7.75±0.02	8.80±0.01	8.00±0.06	8.10±0.02	8.05±0.05	7.00±0.05
Pipradrol	9.71	9.69±0.04	8.21±0.04	8.43±0.05	9.30±0.03	7.73±0.03	9.90±0.05	9.76±0.04	8.50±0.04
Benzphetamine	6.60	6.71±0.03	5.43±0.05	6.03±0.04	6.51±0.02	5.06±0.02	7.14±0.04	6.83±0.06	5.70±0.05
Normethadone	9.20	8.75±0.04	8.11±0.06	8.24±0.02	8.80±0.03	7.00±0.06	9.12±0.05	9.01±0.05	8.02±0.10
Fentanyl Citrate	7.34	7.35±0.01	7.04±0.03	5.75±0.03	4.75±0.05	4.91±0.08	5.78±0.04	5.60±0.15	3.70±0.05
Methadone	8.25-9.64	9.16±0.01	8.05±0.05	9.00±0.06	9.08±0.01	7.81±0.01	9.50±0.04	9.40±0.03	8.15±0.06
Piritramide	8.50	9.01±0.03	7.51±0.04	8.00±0.02	8.92±0.01	7.40±0.05	9.29±0.02	9.23±0.06	8.18±0.04
Norpipanone HCl	8.50	9.02±0.01	8.00±0.02	8.10±0.01	9.00±0.04	7.20±0.03	9.24±0.05	9.18±0.07	8.22±0.06
Dipipanone HCl	8.5-9.08	9.10±0.08	7.80±0.03	8.20±0.03	9.04±0.02	6.70±0.10	9.36±0.08	9.32±0.10	8.31±0.04

4.1.3 DISCUSSION

4.1.3a Influence of Temperature on pKa

The effect of temperature on the pKa of organic bases has been investigated by different workers, and nitrogenous bases are said to be highly temperature sensitive (58-60).

Perrin proposed a general equation governing the temperature variation for the ionization of a base or an acid as:

$$-\frac{d(pKa)}{dT} = \frac{pKa}{T} + \frac{\Delta S^{o}}{2.303RT} = \frac{pKa+0.218\Delta S^{o}}{T} \qquad ...4.3$$

and for monoacidic bases the equation becomes:-

$$-\frac{d(pKa)}{dT} = \frac{pKa - 0.9}{T} \qquad ...4.4$$

where; R = Universal Gas Constant

T = absolute temperature (°K)

ΔS^{0} = entropy change

In this work, pKa has been determined at 20°C and 40°C. Table 4.2, because these were the temperatures used for chromatographic analysis. Equation 4.4 has been used to predict pKas at 40°C and plots of predicted pKa versus experimental pKa at 40°C under different solvent conditions are given in Figures 4.1-4.6. The results indicate that the relationship

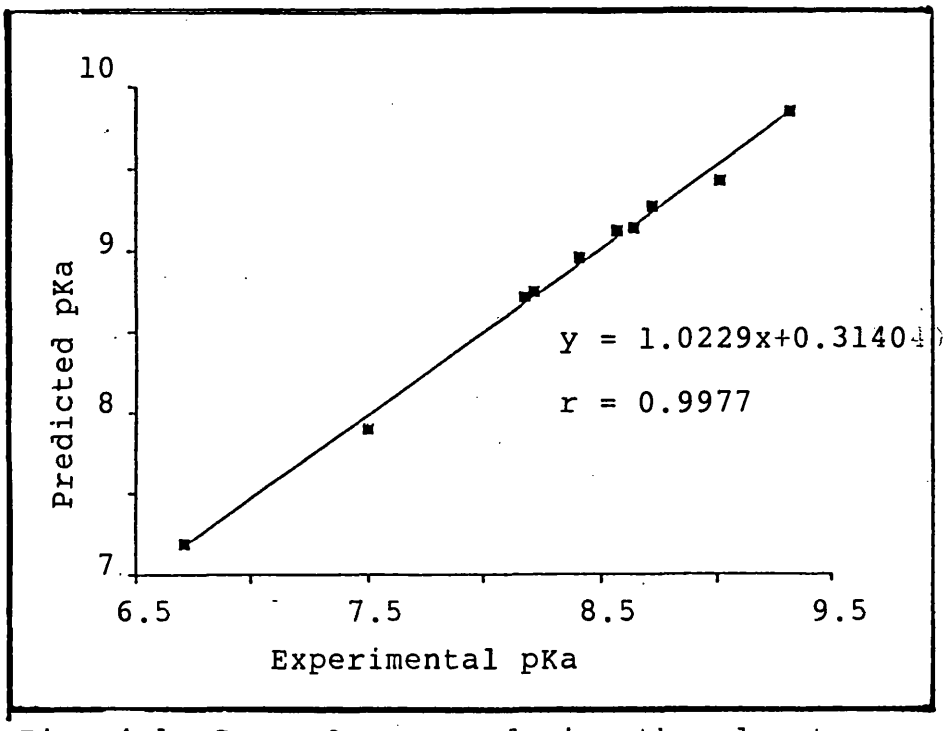


Fig. 4.1. Group A compounds in ethanol-water (50; 50% v/v) at 40°C

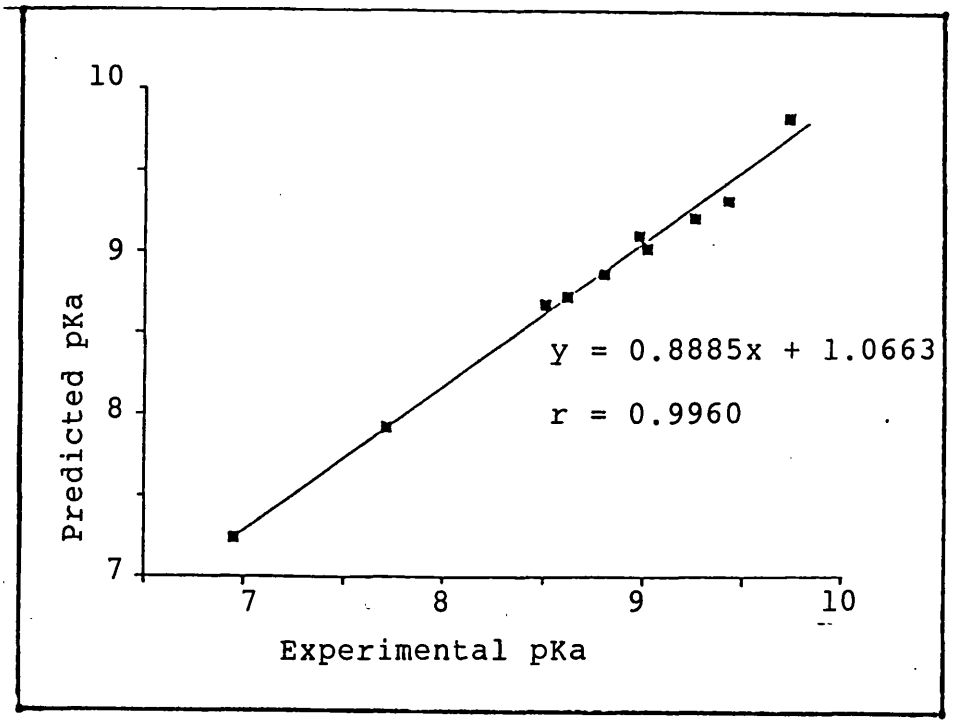


Fig. 4.2. Group A compounds in 0.02 M KCl solution at 40°C

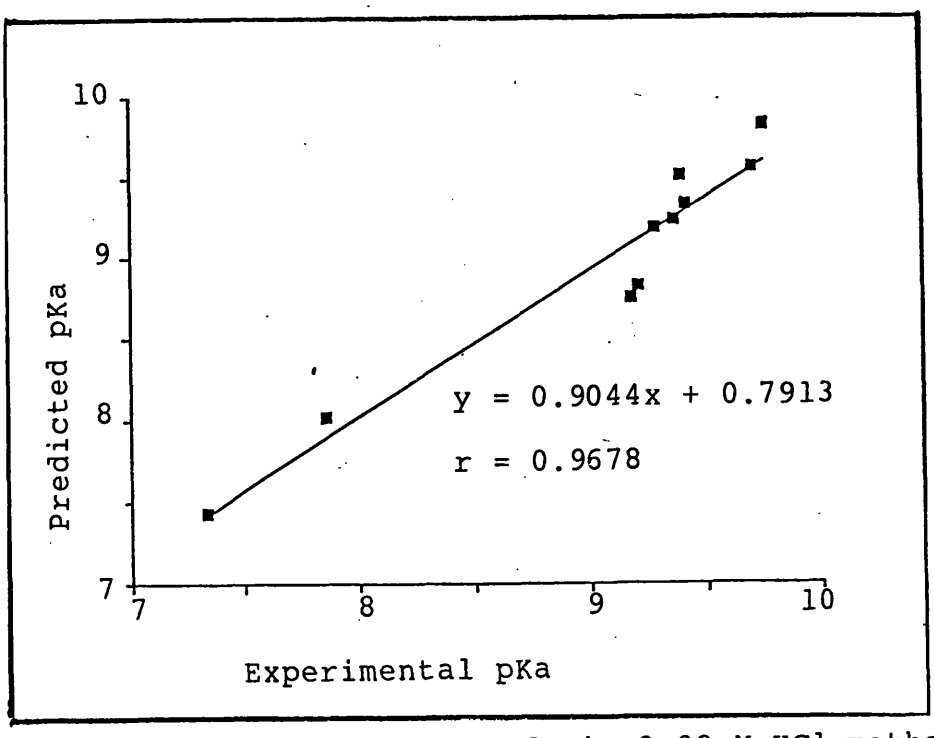


Fig. 4.3. Group A compounds in 0.02 M KCl-methanol-acetonitrile (90; 5; 5 % v/v/v) at 40°C

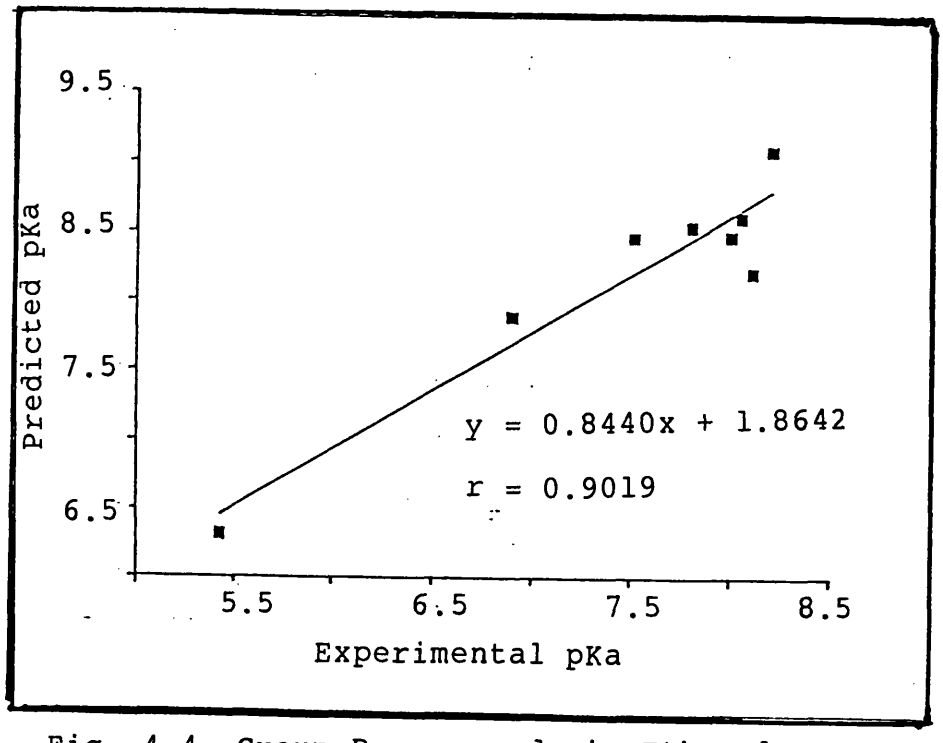


Fig. 4.4. Group B compounds in Ethanol-Water (50; 50 % v/v) at 40°C

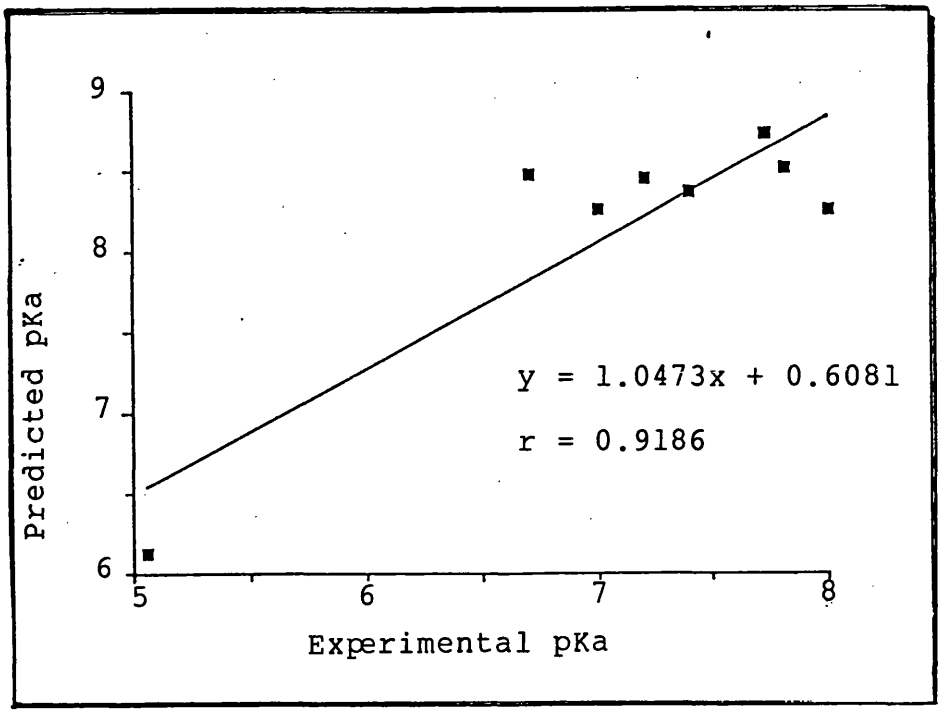


Fig. 4.5. Group B compounds in 0.02 M KCl at 40°C

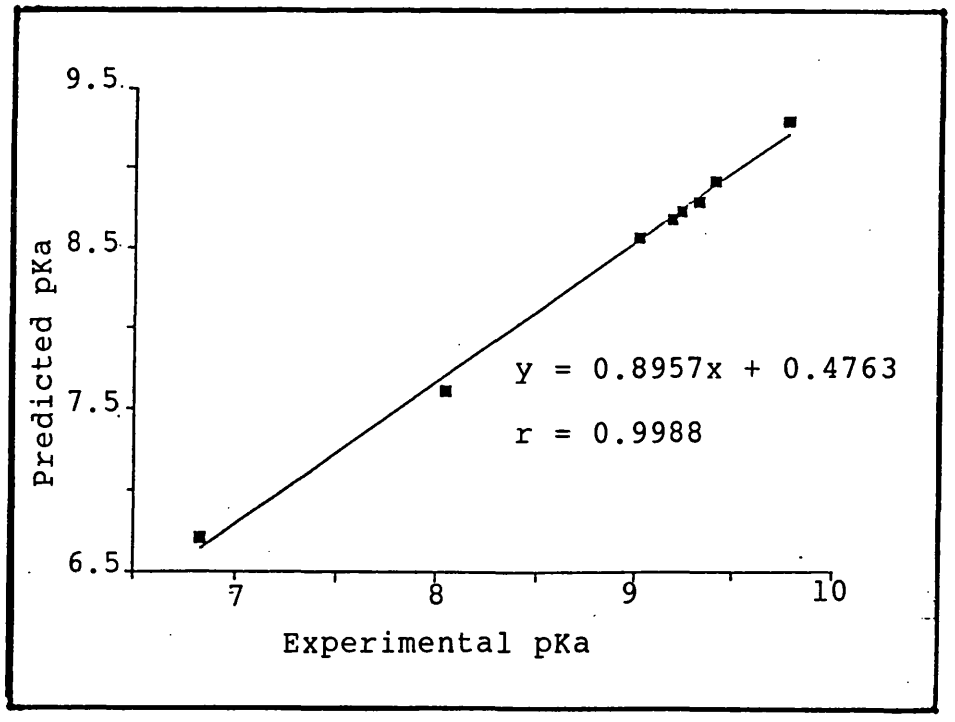


Fig. 4.6. Group B compounds in Phosphate buffer - propan-2-ol - acetonitrile (65; 17.5; 17.5 % v/v/v) at 40°C

between analyte pKa and temperature is a linear one ($r = 0.9186$ to 0.9988) and that the relationships are similar for the solvent conditions examined (slopes = 0.8885 to 1.0663).

An examination of the rate of pKa change per °C temperature change ($\frac{dpKa}{dT}$) for each compound is given in Table 4.5a. This shows that all the compounds give a pKa decrease with an increase in temperature, as reported by previous workers (58, 59), and that the rates of pKa decrease per °C are generally between 0.02 and 0.03. Among Group A compounds, phendimetrazine has the lowest pKa in each of the conditions, followed by phenmetrazine, also the $\frac{dpKa}{dT}$ values are low namely 0.0230 and 0.0256 respectively. An examination of their structures (Figure 2.1B) shows that they both have cyclic nitrogen and cyclic oxygen (morpholine group) which make them weaker bases than other members of the group. Fentanyl citrate in Group B seems not to have any consistent behaviour under these experimental conditions, and has been excluded from the statistical calculations above. From its structure (Figure 2.1B) one would expect it to have two pKa values.

Table 4.5a. Rate of analyte pKa change with temperature, for individual compounds

COMPOUNDS Group A	Conditions: 1 and 2	 4 and 5	 6 and 7
p-OH-Norephedrine	0.0286	0.0285	0.0288
p-OH-Amphetamine	0.0287	0.0287	0.0290
p-OH-N-Methylamphetamine	0.0295	0.0292	0.0304
2-Phenylethylamine	0.0301	0.0297	0.0305
Dexamphetamine	0.0306	0.0304	0.0309
Methylamphetamine	0.0312	0.0309	0.0318
Phenmetrazine	0.0256	0.0257	0.0261
Phendimetrazine	0.0230	0.0232	0.0239
Mephentermine	0.0328	0.0327	0.0327
Chlorphentermine	0.0302	0.0300	0.0316
Group B	**Conditions: 1 and 2**	** 4 and 5**	** 8 and 9**
Pethidine	0.0260	0.0270	0.0246
Pipradrol	0.0300	0.0287	0.0307
Benzphetamine	0.0198	0.0191	0.0213
Normethadone	0.0268	0.0270	0.0281
Fentanyl	0.0220	0.0131	0.0167
Methadone	0.0282	0.0279	0.0294
Piritramide	0.0277	0.0274	0.0286
Norpipanone	0.0277	0.0276	0.0285
Dipipanone	0.0280	0.0278	0.0289

For a description of the conditions used, see Table 4.2.

Table 4.5b. Average rate of analyte pKa change with temperature

GROUP	Conditions: 1 and 2	4 and 5	6 and 7
A:			
Mean	0.0290	0.02890	0.02424
Standard deviation	0.0028	0.00269	0.01154
	Conditions 1 and 2	4 and 5	8 and 9
B:			
Mean	0.0262	0.0250	0.0263
Standard deviation	0.00326	0.0053	0.0046
Overall Mean (Groups A & B) =	0.0267		
Relative Standard Deviation =	7.10%		

Comparison of the means obtained under identical conditions shows that Groups A and B behave similarly. Comparison of the means obtained under different conditions are also similar, so that an average value for the dependence of pKa upon temperature has been calculated (Table 4.5b). The figure of 0.027 represents the average fall in pKa value per °C rise for the nineteen compounds examined here, and could be a useful guide when considering chromatographic optimisation.

In an attempt to explaim more accurately the effect of temperature upon pKa, the results in Table 4.5 were used to calculate ΔS^{o} values using equation

4.3, and then an average ΔS^{o} value obtained which could be used to predict dpKa. This approach failed for all the results in Table 4.5, except for Group A compounds under Conditions 1 and 2, for which the relevant data is given in Table 4.6. Even here, the correlation coefficient between predicted dpKa and experimental dpKa was only 0.8160 and so this is not a satisfactory approach. This is probably because the ΔS^{o} values are dependent upon the solvent compositions in which they are determined, and should be determined experimentally. This was the approach used by Perrin (60), who averaged the experimental data from twelve compounds (range -8.1 to 1.7) to obtain the figure of -0.9 used in equation 4.3.

Table 4.6. Calculated entropy change (ΔS^{o}) for Group A compounds

COMPOUNDS	ΔS^{o}_{calc}	$dpKa_{calc}$	$dpKa_{expt}$
p-OH-Norephedrine	31.97	1.07	1.11
p-OH-Amphetamine	31.17	1.08	1.10
p-OH-N-Methylamphetamine	32.80	1.09	1.14
2-Phenylethylamine	32.69	1.11	1.15
Dexamphetamine	32.63	1.12	1.16
Methylamphetamine	23.79	1.13	1.04
Phenmetrazine	22.58	1.02	0.91
Phendimetrazine	28.08	0.96	0.94
Mephentermine	31.76	1.16	1.19
Chlorphentermine	29.24	1.07	1.10

Average ΔS^{o}_{calc} = 29.67

4.1.3b Influence of organic solvents on pKa

Although it is desirable to measure dissociation constants in pure water, this is often not employed either because the compounds are poorly soluble in water or because other solvents are more appropriate to other studies, e.g. pharmaceutical formulation. In this work water and ethanol have been used as typical

solvents for pKa determinations whilst methanol, propan-2-ol and acetonitrile have been included in volumetric proportions that have been chosen for chromatographic reasons. An examination of some of the properties of these solvents that could affect the dissociation of a compound are shown in Table 4.7.

Table 4.7. Properties of Solvents

SOLVENT	Dipole Moment μ, D (g)	Dielectric Constant ϵ (20°C)
Water	1.85	80
Methanol	1.70	33
Ethanol	1.69	25
Propan-2-ol	1.66	22
Acetonitrile	3.92	38.8

Since alcohols and water contain an -OH group, they have many properties in common. Water is a very remarkable substance and one of its important properties is self-ionization:-

$$H_2O + H_2O \rightleftharpoons H_3O^+ + OH^- \qquad ...4.5$$

In pure water, the concentrations of H_3O^+ and OH^- are low, only 10^{-7} mole L^{-1}. Alcohols also undergo self-dissociation, but not to the same extent as water, so

that for methanol, the ion product

$$2CH_3OH \rightleftharpoons CH_3OH_2^+ + CH_3O^- \qquad \ldots 4.6$$

is only 1.2×10^{-17} M^2 whilst that of water is 1.0×10^{-14} M^2. The reduced value for alcohols are due largely to their dielectric constants (61) compared to water, i.e. it takes more energy to separate ions in alcohols, in other words, as the dielectric constant is reduced, the electrostatic interactions between ions increase (62). This phenomenon can be explained by applying Coulomb's law of electrostatic force expressed in terms of the work required to separate two ions of opposite sign in the medium (59).

Work is expressed as $\frac{e^2}{\varepsilon . r}$ ... 4.7

where, e = 4.8×10^{-10} electrostatic units

r = distance between charges (2×10^{-8} cm)

and ε = dielectric constant

If we therefore calculate the work necessary to separate ions in water and in methanol, we obtain values of 1.4×10^{-13} erg and 3.49×10^{-13} erg respectively. This means that for organic acids, pKa values will be increased, whilst for organic bases, pKa values will be reduced in methanol compared to water. This effect would also apply to other solvents,

including those in Table 4.7. Data taken from Tables 4.3 and 4.4 confirms this for the effect of ethanol and is shown in Table 4.8. The dielectric constant of a mixture of solvents is calculated from the sum of the volume ratios of the dielectric constants of the individual solvent components (63), this effect is shown in Table 4.8. The dielectric constant values in Table 4.8 confirms that the effect of ethanol would be to reduce pKa values.

Table 4.8. Effect of dielectric constant of a mixture of solvents on pKa

COMPOUNDS	0.02 M KCl (100% v/v) ϵ_{calc} (80.0)* pKa (20°C)	Ethanol-0.02 M KCl (50; 50% v/v) ϵ_{calc} (52.5) pKa (20°C)
p-OH-Norephedrine	9.25	7.62
p-OH-Amphetamine	9.30	7.91
p-OH-N-Methylamphetamine	9.45	8.22
2-Phenylethylamine	9.61	8.35
Dexamphetamine	9.82	8.41
Methylamphetamine	9.96	8.62
Phenmetrazine	8.43	8.90
Phendimetrazine	7.70	6.25
Mephentermine	10.48	8.95
Chlorphentermine	9.70	8.10
Pethidine	8.80	7.75
Pipradrol	9.30	8.43
Benzphetamine	6.51	6.03
Normethadone	8.80	8.24
Piritramide	8.92	8.00
Norpipanone	9.00	8.10
Dipipanone	9.04	8.20

* Dielectric constant of KCl solution is assumed to be the same as that of water.

Table 4.9. Effect of temperature on pKa

COMPOUNDS	pKa (20°C)	pKa (40°C)	pKa (20°C)	pKa (40°C)	pKa (20°C)	pKa (40°C)
*CONDITIONS	(1)	(2)	(3)	(4)	(5)	(6) (7)
p-OH-Norephedrine	9.29	8.18	9.25	8.51	9.34	9.18
p-OH-Amphetamine	9.32	8.22	9.30	8.62	9.41	9.21
p-OH-N-Methylamphetamine	9.55	8.41	9.45	8.80	9.80	9.28
2-Phenylethylamine	9.72	8.57	9.61	9.01	9.85	9.36
Dexamphetamine	9.88	8.72	9.82	9.25	9.96	9.41
Methylamphetamine	10.05	9.01	9.96	9.42	10.21	9.70
Phenmetrazine	8.41	7.50	8.43	7.71	8.54	7.86
Phendimetrazine	7.65	6.71	7.70	6.95	7.91	7.33
Mephentermine	10.51	9.32	10.48	9.73	10.48	9.75
Chlorphenetermine	9.74	8.64	9.70	8.97	10.15	9.39

* For Conditions Refer to Table 4.2.

The effect of temperature on dielectric constants is to reduce their values as temperature increases (40), so that the organic solvent effect on pKa is enhanced as temperature is increased. This can be seen also in Table 4.9.

4.1.3c Effect of potassium chloride on pKa

Potassium chloride is sometimes used as an indifferent electrolyte. Any electrolyte in aqueous phase will dissociate and be randomly scattered in the organic solute. Protonation of an organic base by H_3O^+ will be hindered due to the presence of inorganic anions, therefore more H_3O^+ is required to overcome their effect i.e. a lower pH is required to protonate the organic base, and thus pKa is reduced. This is demonstrated in the results obtained in this work which are shown in Table 4.10.

It is interesting to note that 50% v/v 0.02 M KCl has approximately the same effect on ionization constants as 50% ethanol, especially for Group A compounds in this work.

Table 4.10. Effect of potassium chloride on pKa

	Ethanol-Water (50; 50% v/v) pKa (20°C)	Ethanol-0.02 M KCl (50; 50% v/v) pKa (20°C)
p-OH-Norephedrine	9.29	7.62
p-OH-Amphetamine	9.32	7.91
p-OH-N-Methylamphetamine	9.55	8.22
2-Phenylethylamine	9.72	8.35
Dexamphetamine	9.88	8.41
Methylamphetamine	10.05	8.62
Phenmetrazine	8.41	8.90
Phendimetrazine	7.65	6.25
Mephentermine	10.51	8.95
Chlorphentermine	9.74	8.10
Pethidine	8.40	7.75
Pipradrol	9.69	8.43
Benzphetamine	6.71	6.03
Normethadone	8.75	8.24
Methadone	9.16	9.00
Piritramide	9.01	8.00
Norpipanone	9.02	8.10
Dipipanone	9.10	8.20

4.2 Examination of the Partition Coefficient (log P) of the Compounds Investigated

The partition coefficient of a solute is a measure of its distribution between two immiscible liquid phases (40, 64), most commonly n-octanol and water. It is also a measure of solute lipophilicity, which is an important property of any drug substance because of its influence on solute biological properties.

The influence of a drug substance can only be obtained if it can reach the active site by a transfer process that involves passage through both hydrophilic and lipophilic barriers in the biological system. Therefore, the hydrophobic character of drugs is very important in drug design. Experimentally, log P values are determined under aqueous conditions which ensure that the compound, if it is an ionisable compound, is fully non-ionised. However care is required in the selection of literature log P values because this is not always the case.

In reversed-phase HPLC, retention of a solute is described mainly as a function of its solvophobic interaction between an aqueous-organic mobile phase and a non-polar stationary phase (33, 44-47). This is essentially the same as the partitioning interaction between water and an immiscible organic solvent such as n-octanol. Although solute retention mechanisms may be

complex and varied, it is a fact that in RP-HPLC solute retention can be correlated to their n-octanol-water partition coefficients. Attempts have been made to obtain substituent constants, for various functional groups (65-67), and this approach to the calculation of log P values for solutes, in general, is very useful.

However, an examination of literature experimental log P values indicates that considerable care is required in the selection of values. Table 4.11 illustrates the variation in log P values quoted for a single compound, pethidine, (literature pKa = 8.7 at 20°C) when measured at slightly different pH values. All these values were obtained by the same author.

An examination of the Database (68) for log P values showed that for most of the compounds under examination in this study, values were not available. It was therefore considered appropriate to experimentally determine these values, and in particular to determine them in the chromatographic mobile phases that had been optimised in Section 3, because Table 4.11 clearly shows the importance of aqueous phase composition.

Table 4.11. Variation in experimental log P values for pethidine, for water-n-octanol, from ref. 4.13

Aqueous conditions given by author	log P
pH 7.1	1.30
pH 7.4, phosphate buffer, not ion-corrected	1.59
pH 7.5, phosphate buffer, not ion-corrected	1.68
pH 7.6, where 2N in side chain, some di-protonation probable	1.77
pH 7.7, not ion-corrected	1.86
pH 7.7, ion-corrected	2.72

4.2.1 EXPERIMENTAL

The method used in this work is that described by Leo et al. (69), but using our own chromatographic mobile phases as described in Section 2.7 as the aqueous phase. The performance of this method and equipment was tested using fentanyl citrate at room temperature (21°C) and pH 7.4. An average value of 2.36 (n = 2) was obtained which compared favourably with the literature value of 2.30 at pH 7.4 (66). The method was therefore used for all the compounds in this thesis and the results are given in Table 4.12.

Table 4.12. Experimentally determined Log P values

Group A	
p-OH-Norephedrine	0.18
p-OH-Amphetamine	0.20
p-OH-N-Methylamphetamine	0.22
2-Phenylethylamine	0.22
Dexamphetamine	0.63
Methylamphetamine	1.69
Phenmetrazine	1.02
Phendimetrazine	2.06
Mephentermine	0.82
Chlorphentermine	1.10
Group B	
Pethidine	0.72
Pipradrol	0.35
Benzphetamine	0.54
Normethadone	1.41
Fentanyl	1.51
Methadone	1.98
Piritramide	2.15
Norpipanone	2.76
Dipipanone	2.94

4.2.2 DISCUSSION

These values are greatly influenced both by the pH of the aqueous phase and the presence of organic solvents as chromatographic modifiers. For Group A compounds the conditions were 0.02 M phosphate buffer pH 5.4 - methanol-acetonitrile (90; 5; 5% v/v/v) whilst for Group B compounds the conditions were 0.02 M phosphate buffer, pH 4.1 - propan-2-ol-acetonitrile (65; 17.5; 17.5% v/v/v). The influence of pH can be incorporated into partition coefficient determinations by using the Apparent Partition Coefficient (Papp)

where $$Papp = P.Mn \qquad ...4.8$$

where Mn = fraction of molecules present in the non-ionised form.

For a monoacidic base,

$$Mn = \frac{1}{[1 + 10^{pKa-pH}]} \qquad ...4.9$$

So that

$$P_{app} = \frac{P}{[1 + 10^{pKa-pH}]} \qquad ...4.10$$

It is assumed that solute ions are not extracted into the solvent layer. However, this equation would need further modification in order to take into account the presence of chromatographic modifiers.

4.3 Relationships between hydrophobic parameters (log P), dissociation constants (pKa) and solute capacity ratios (k')

The purpose of determining log P and pKa values in chromatographic mobile phases was in order to make better predictions of solute retention. Using the data obtained, attempts were made to find correlations wherever possible.

4.3.1 RELATIONSHIP BETWEEN EXPERIMENTAL LOG P AND LOG k'

Since the experimental log P values were determined in the chromatographic mobile phases, it was anticipated that a relationship with solute retention should be observed. Fig. 4.7 shows that for Group B compounds a linear relationship was observed, $r = 0.988$, $n = 7$, but no similar relationship was found for Group A compounds. The reasons for this are not understood.

4.3.2 RELATIONSHIP BETWEEN EXPERIMENTAL k' AND PREDICTED k'

In the work of Hafkenscheid and Tomlinson (42), a description of the relationship between the retention of basic solutes and the mobile phase pH was represented by the following equation:-

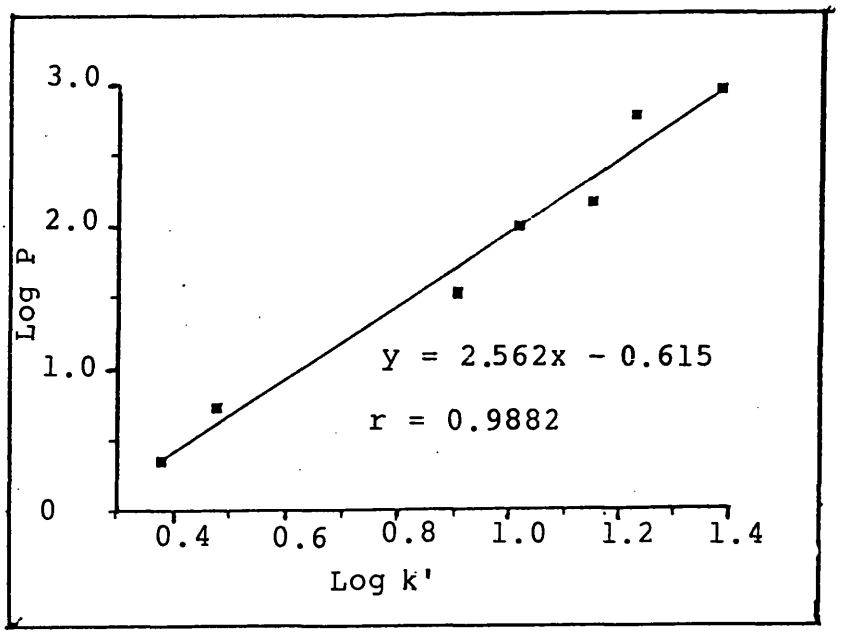


Fig. 4.7. Relationship between log P and log k' of Group B compounds

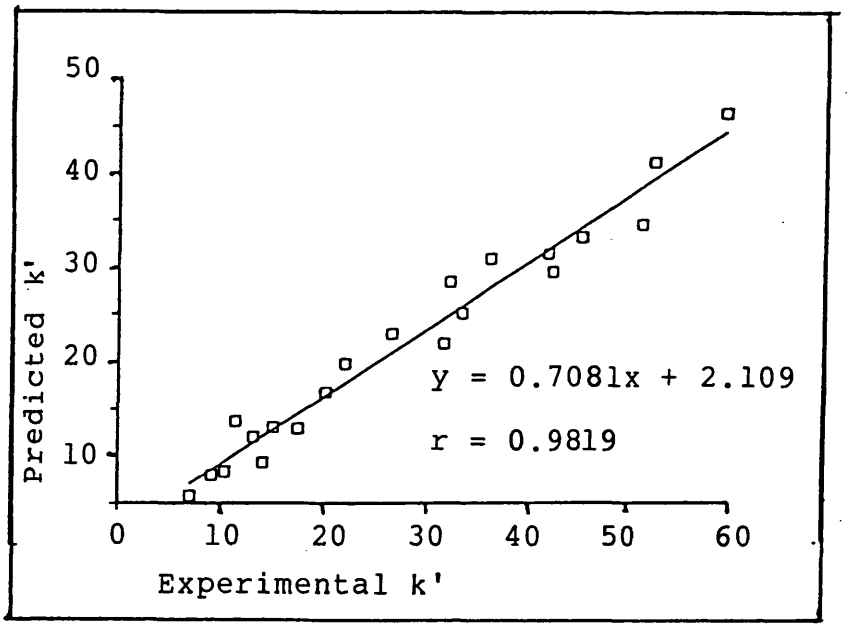


Fig. 4.8. Relationship between experimental k' and predicted k' of Group B compounds

$$k' = [1 + 10^{(pKa-pH)mob}]^{-1}.k^{o}+[1+10^{(pH-pKa)mob}]^{-1}.\ k^{+}$$

...4.11

where k' = non-logarithmic capacity factor of the chromatographed species, be it the solute, its uncharged form or its monoprotic charged form

pKa = solute pKa under mobile phase condition

pH = mobile phase pH

mob = mobile phase

k^0 = capacity factor in uncharged form

k^+ = capacity factor in charged form.

This equation was applied to calculate k^o and consequently predict k' at the different experimental pHs of 5.6, 6.2 and 6.8. A relationship was then sought between predicted k' and experimental k'. This is expressed in Figure 4.8. A correlation coefficient of 0.9819, n = 21 was obtained, only about three or four points could be considered as outliers.

4.3.3 CONCLUSIONS

The results obtained in the determination of pKa suggest that under experimental conditions used for the chromatographic analysis of Groups A and B compounds in this work, pKa values are lower than literature values. For Group A (n = 7) a mean value of

-0.768 (range -0.45 to -1.20) and for Group B (n = 8) a mean value of -0.735 (range -0.10 to -1.70) were obtained. For Groups A and B, the mean reduction in pKa value was -0.75.

As expressed in Section 4.1, the experimental pKa values obtained are not adequate to explain the continuous fall in capacity ratios of these compounds at pH below 4.0. It would appear that other factors apart from the interaction of the protonated solutes with the bonded phases are responsible for any further retention of the solutes below pH 4.0.

Iler (130) had pointed out that basic to the adsorption or exchange of cations on silica is the degree of ionization of the SiOH groups on the surface. The pKa of these SiOH groups varies from 6.5 at 0% neutralisation to about 9.2 at 50% neutralisation. Complete ionization occurs only at the pH just short of the point where silica dissolves and then only in the presence of a strong salt solution.

Mant et al. (131) have monitored free silanols on reversed-phase supports using peptide standards and found considerable differences between commercial silicas when monitored at pHs 2, 4.5 and 7. In general they consider a pH of 2.0 is required to suppress silanol ionization to minimise ionic interaction with basic peptide residues, but that this pH does not necessarily eliminate silanol effects because it depends upon the type of silica used. Silanols are

negatively charged above pH 3.5 - 4.0, resulting in strong ionic interactions with basic peptide residues.

The interactions between protonated amines in this study and the free silanol group of the chromatographic packing materials used in this work is analogous to the interactions described by Mant et al. This is evident when Figures 3.1 and 3.2 are compared. It is noted in Figure 3.1 that capacity ratios are still falling steeply at a pH of 5.6 while in Figure 3.2 at pH 4.0 capacity ratios are more or less levelling out. Also in Figure 3.18 with pH 3-4, capacity ratios have almost levelled out.

In theory, one would expect the fall in k' values with reduction in mobile phase pH to reflect the increase in protonation of a base with fall in pH, i.e. a titration curve.

The effect of organic modifiers, buffer salts, and an increase in temperature all contribute to a lowering of pKa values for basic compounds, which for any particular mobile phase pH, means that retention is greater than expected, as shown in Figure 4.9.

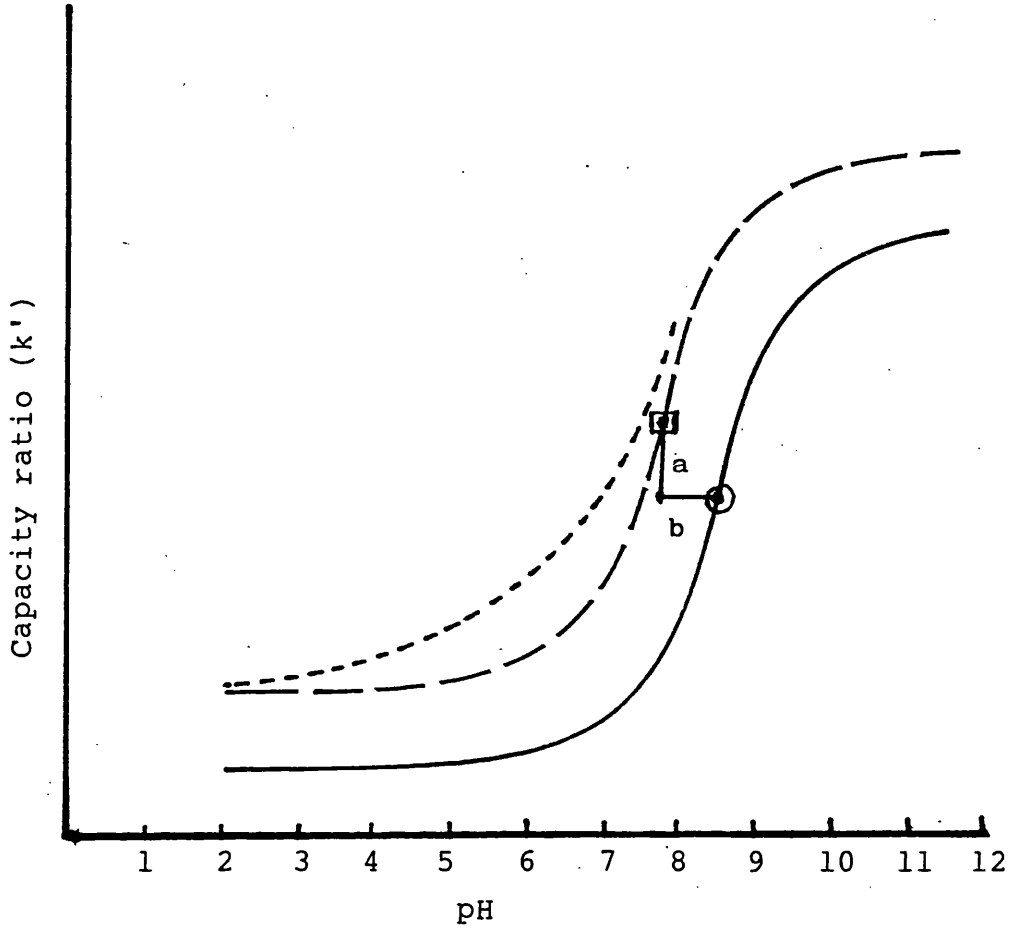


Figure 4.9. Influence of mobile phase components and pH on retention of basic drugs of abuse using silica-based reversed phase H.P.L.C.

-------- : increase in k' due to silanol effects

— — — : increase in k' due to chromatographic conditions

———— : theoretical k' for basic compounds, according to % protonation

⊙ : Mean solute literature pKa (n=15)

⊡ : Mean solute experimental pKa (n=15) in chromatographic phases

a : Nominal increase in k' (∝ to solute log P) caused by change in pKa

b : Experimental average fall in pKa (-0.75)

The fall in retention with pH does not follow the new titration curve however because ionisation of silanol groups above pH 3.5-4.0 is significant, and its effect is to increase solute retention due to ionic interactions. Figure 3.18 shows that for Hypersil-SAS, solute retentions had not reached minimum values at pH = 3.0, so that even when silanol ionisation is minimal then other effects, such as hydrogen bonding may still increase solute retention above the theoretical minimum.

SECTION 5

POST-COLUMN DERIVATIZATION

SECTION 5 POST-COLUMN DERIVATIZATION

For the majority of analyses optimization of the chromatographic separation process is generally more important than the method of detection. However for those compounds having weak UV absorptivity, the problem of how to achieve adequate detection becomes the major concern, especially when the concentration levels expected in plasma are in the $ng.ml^{-1}$ range.

The chemical derivatisation of analytes to enhance detection is not new and has been used for classical UV spectrophotometry and fluorescence so that its application to liquid chromatography was a logical step. A number of publications have provided information on both pre- and post-column derivatization coupled with HPLC (2, 70-83). Pre-column reactions were examined for their possible modification to a post-column system.

UV spectrophotometry, fluorescence and electrochemical detection were considered as possibly suitable for the organic amines in this study. Electrochemical detection was considered because HPLC assays have been described for narcotics (84, 85) and p-hydroxyamphetamine in mouse brain (86). Detection was achieved in each case by oxidation of a phenolic hydroxyl group. This would be suitable for some

metabolites examined in this study but not for most of the analytes. Unfortunately, the detection of tertiary amines (87) has been reported to require a pH > 10, which means that silica based column materials could not be used especially if the mobile phase also needed to be mainly aqueous. The use of polymeric column materials would overcome the pH problem, but could not provide efficient chromatography using predominantly aqueous mobile phases. It is possible that a post-column addition of alkali to raise eluent pH followed by electrochemical detection might provide a suitable method for primary, secondary and tertiary amines, but this would require a great deal of study and was not examined experimentally.

Ultraviolet spectrophotometry and fluorescence have frequently been described for pre- and post-column systems and much attention has been given to primary and secondary amines. However almost half of the analytes considered in this study are tertiary amines and cannot be derivatized by any of these reagents.

Reports describing procedures for tertiary amines are very infrequent, although Kudoh et al. (88) developed a post-column system using acetic anhydride in citric acid to produce a red colour specific for tertiary amines. However the chromatographic selectivity was poor, tridecylamine and tridodecylamine were not resolved, the chromatographic conditions were

normal phase, moisture reduced the colour produced and the post-column reaction needed a temperature of 120°C.

It was decided that ion-pair formation would be the most suitable initial approach to enhanced detection in a post-column system because:

a) All amines, when protonated, readily form ion-pairs with suitable reagents,

b) ion-pair formation is almost instantaneous and occurs at room temperature, so that adverse effects upon the analyte bands eluting from the HPLC column would be minimal.

Post-column derivatization systems have some advantages over pre-column derivatization methods, because in the post-column situation, the conditions of the reaction, (time, temperature, environment) are closely controlled by the dimensions and configuration of the equipment, so that the derivatization can be highly reproducible. Furthermore, it is not necessary for the reaction to go to completion. Time saved on sample preparation can be used to increase sample throughput. The most important disadvantage of post-column systems is the additional cost and complexity of the instrumentation.

Derivatization reactions are carried out in the post-column mode by the addition of reagents to the eluent from the HPLC column either by segmented flow analysis (SFA) or flow injection analysis (FIA). These

techniques have been adequately summarized (89-104) and their relative merits have been debated (105-109). Even though both employ similar chemical reactions, to give detectable end products, they differ in the 'mechanics' of sampling and use different approaches to achieve suitable precision and accuracy. Segmented flow injection analysis is potentially more sensitive than non-segmented and therefore has generated much interest recently. Primarily, it is the small sample volume, the low reagent consumption (110) and the high sampling frequency that attracts analytical chemists. Since extraction is widely used in pharmaceutical and clinical analysis to separate drugs or drug metabolites, it was logical to attempt to apply this principle as a post-column step to this study. The aim being to achieve better economy of extraction method with respect to time and solvent consumption.

All post-column reactors are continuous-flow devices (5). An ideal post-column reactor should be designed to permit chemical reactions to be performed reproducibly, carried far enough to completion for sensitivity, and yet, introduce little extra column band-broadening. Because of diffusion processes that occur in fluid solution, all designs introduce some measure of band broadening. This is largely due to laminar flow and the interaction of the fluid stream with the walls of the reactor tubing.

Common reactor types are:

a) Tubular reactor - This is the simplest form of reactor consisting of an open-tubular system in which the post-column reagents are pumped into the eluate stream, mixed in a low-volume tee, and reacted in a coil of appropriate length and diameter, typically as described by Deeler and co-workers (111).

b) Bed reactor - This can be a packed chromatographic column that operates under zero retention characteristics. It has been suggested by Hilby (112), that band broadening can be minimized by using columns packed with very small particles, such as glass (Balotini) beads (0.15-0.2 mm).

c) Segmented Stream Reactor - Here the flowing stream is a tubular system segmented with uniformly spaced air bubbles or with an immiscible solvent so that analyte dispersion is considerably reduced.

Post-column chemistry is a very important part of the post-column derivatization process. The chemistry can be very simple, for example a 0.5 pH change (113-115) can be sufficient for the reaction. In this case a simple open-tubular reactor with a short path length is sufficient, since reagents need only to be mixed. A segmented stream reactor permits the use of a wider range of reagents to the HPLC eluent, and depends upon the derivatized analyte being extracted into an

immiscible organic solvent phase leaving the reagents in the HPLC eluent. This is essential in order to avoid massive UV or fluorescent background signals overloading the detector.

The success or failure of this approach depends upon the availability of a continuous flow aqueous-organic solvent phase separator, and its efficiency in providing to the detector a supply of organic solvent that is completely free of water droplets. At the time of this study no phase separator designed for the low flowrates employed in HPLC was commercially available, and publications described either modifications to the Technicon separator (120) which was designed for automated amino acid analysers, or described various in-house designs. A separator made by Kinkel and Tomlinson (5) was kindly loaned for testing and then a copy made at Bath University. This design was later modified by Dr. T.M. Jefferies in order to improve its performance at higher flowrates. Both separators have been described in Section 2.

5.1 INVESTIGATION OF THE PERFORMANCE OF TWO PHASE SEPARATORS

Both separators were tested for their ability to supply to the UV detector a continuous flow of chloroform-pentan-1-ol (90:10; % v/v) that was totally free from water droplets. The aqueous phase was the

HPLC eluent developed for Group A compounds using a Hypersil SAS column, namely methanol-acetonitrile-0.025 M aqueous phosphate buffer, pH 5.4 (5; 5; 90;% v/v/v). The organic solvent - aqueous phase volume ratio was maintained at (2:1) and the flowrates increased to determine the maximum flowrate at which clean organic solvent could be delivered to the UV cell. The results are summarised in Fig. 5.1.

The original phase separator (PSI, Fig. 2.7) had a single channel (1 x 1 x 17 mm) cut into the glass and PTFE blocks so that they coincided exactly. The major aqueous and organic outlets were situated in a small semi-circular chamber (2 mm radius) cut in each channel. Beyond the combined circular chamber, a single channel outlet permitted excess water and organic solvent to be pulled through the separator, improving the flowrate. Fig. 5.1 shows that the proportion of clean organic solvent to the UV cell rose to a maximum of 60% at a total organic flowrate of 1.5 ml min^{-1}. Above this flowrate droplets of water also entered the UV cell producing an unacceptably noisy baseline.

It was found that a constant flow of organic solvent, water-droplet free, could be obtained for the UV cell more readily when the proportion of organic phase to HPLC aqueous phase was about 2;1. For an HPLC column of 4.6 mm I.D. the most commonly used flowrate is 1 ml min^{-1}. The volume of aqueous reagent added

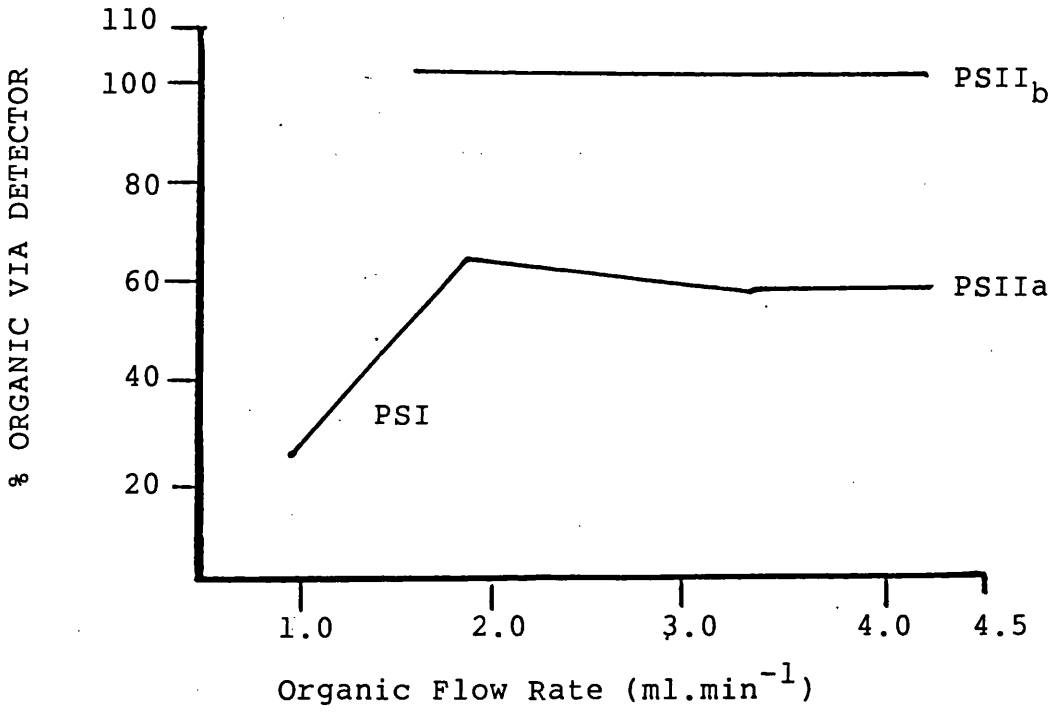


Figure 5.1. Efficiency of the phase separator at the different flow rates (Organic: Aqueous, 2:1).

PSI - Phase separator I

PSII$_a$ is without cleaning channel, while PSII$_b$ is with the cleaning channels connected.

post-column should be kept to a minimum to minimise analyte band broadening,and would often be about 0.25 ml min^{-1}. This meant that an organic flowrate of about 2.5 ml min^{-1} would be required. Since this exceeded the capacity of the phase separator tested, a modified version, PSII was designed by Dr T.M. Jefferies and made in the University. The three major differences in the design, Fig. 2.8, were:

a) The dimension of the main channel was increased to 1 x 1 x 32 mm,

b) the addition of a second channel (1 x 1 x 24 mm) to remove water droplets from the organic phase,

c) the addition of a third channel (1 x 1 x 24 mm) to remove organic solvent droplets from the aqueous phase. This meant that the aqueous phase could also be cleaned and used as the phase monitored by the UV cell, if required.

Fig. 5.1 shows that when only the main channel was used as in PSIIa, the proportion of organic phase passing through the detector was consistently about 60%, similar to the best performance of PSI. However it was able to produce this over the entire range tested, namely 1.5 to 4.25 ml min^{-1}. When the second channel was connected, PSIIb, it was found that all the organic solvent passed through the detector cell, over the same range of organic phase flowrate. Since it was not

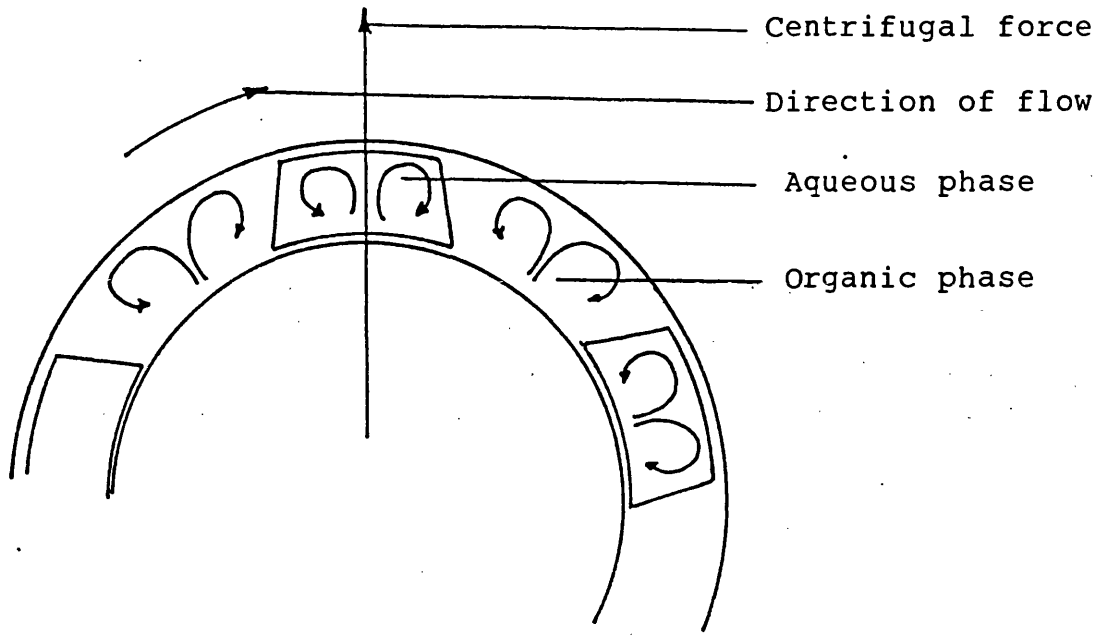


Figure 5.2. Organic and Aqueous segments flowing through a PTFE coil.

Table 5.1 The effect of coil diameter on segment stability for Phase Separator II.

Coil diameter (cm)	No. of segments per 5 cm entering coil (a)	No. of segments per 5 cm leaving coil (b)	Segment Stability Factor (C)*
5.0	30	17	0.57
6.0	30	23	0.75
6.7	30	30	1.00

* $C = \frac{b}{a}$

expected that higher flowrates would be needed, in view of the cost of high quality organic solvents, the capacity of the phase separator was not tested any further.

Since the completion of these studies, a phase separator designed for HPLC has become commercially available from the Dept. of Analytical Chemistry, Free University, Amsterdam. Its design and performance have been described by de Ruiter et al. (116), and works on the same wetting principle as the Kinkel and Tomlinson model (5) used in this thesis. It has a single channel, 10 mm long, total volume 40 µl, and the authors recommend that it is used with an aqueous- organic ratio of (1:1 % v/v) at 1 ml min^{-1} of each phase. Under these conditions, it will deliver about 35% of the organic phase to the detector cell.

5.2 EFFECT OF EXTRACTION COIL DIAMETER AND FLOW RATE ON SEGMENT STABILITY

The extraction of derivatized analytes into an organic phase from an aqueous phase can be achieved simply and continuously by segmentation of the two phases, followed by their passage through a tubular coil. It has been found in this study that a PTFE 'Tee' piece with an internal diameter of about 0.8 mm makes a satisfactory segmentor and when connected to PTFE tubing of 0.8 mm I.D. produces segments 2-4 mm wide for

aqueous-organic phases that are in a volume ratio 1:2.

In order to achieve efficient extraction, the tubing needs to be coiled to introduce centrifugal force effects upon the moving segments. This force is at right angles to the direction of liquid flow and so the combined effect is to make the segments thinner and wider (Figure 5.2). If this effect is too great then alternate segments coalesce into single larger segments so that the number of segments per centimetre leaving the coil is less than that entering the coil. This would be detrimental and produce unacceptable analyte band broadening. It is important therefore to determine the smallest coil diameter consistent with segment stability. This is defined as:

$$\frac{\text{number of segments in 5 cm leaving the coil}}{\text{number of segments in 5 cm entering the coil}}$$

and ideally should be 1.0.

It was found that segment stability was related to coil diameter at the flow rates used (0.70-1.30 $ml.min^{-1}$ aqueous + 1.30-2.60 $ml.min^{-1}$ organic), and that good stability could be achieved with a coil diameter greater than 6 cm. Segment stability was studied with phase separator II at ten different flowrates over the range 2.0 to 6.3 $ml.min^{-1}$, and excellent stability ($\simeq$ 1.0) was obtained (Table 5.1). It was noticed that segment size decreased as the flow rate increased, from 1.3 ml at 2.0 $ml.min^{-1}$ to 0.85 μl

at 6.3 $ml.min^{-1}$. Since better liquid-liquid extraction can be expected with smaller segment volumes, this is an unexpected advantage of using high flowrates. However even the 1.3 µl segments can be considered to be satisfactory, considering that most HPLC peaks elute in a volume of at least 1 ml.

5.3 INFLUENCE OF pH AND PENTAN-1-OL ON THE TRANSFER OF NS TO THE ORGANIC PHASE

Naphthalene-2-sulphonate (NS) has been extensively studied as a UV-indicating ion-pair reagent by Crommen et al. (117, 118), and used in straight phase chromatographic conditions. This was achieved by loading a silica column with an aqueous buffered solution of NS and then displacing the aqueous phase between the silica particles with a mobile phase of chloroform-pentan-1-ol. In this system, injected solutes such as alkylamines would form ion-pairs with NS in the aqueous stationary phase within the silica pores, and then transfer by liquid-liquid partition into the mobile organic phase. The alkylamines would be detected as their NS ion-pairs. NS was chosen because it has a very low distribution to the mobile phase, even at pH 2.5, so that it was considered to be suitable not only for amines, but also for amino acids and dipeptides that are in cationic form only at pH < 3. The molar absorptivity (ϵ) of NS is about 1400

at 254 nm (118) which was sufficiently high to obtain detection of the alkylamines down to about 10 ng. Earlier studies by Farrell and Jefferies (2) had found this straight phase system to be difficult to control because of excessive baseline noise and the unpredictable loss of significant volumes of the stationary phase, rendering the system unusable. However, the proven suitability of NS as an ion-pair reagent for amines between aqueous-chloroform phases meant that it could provide a starting point for the development of a post-column ion-pair extraction system, having the same sensitivity, but better stability than the straight phase system.

The transfer of a protonated amine, HA^+, from an aqueous solution containing an excess of an oppositely charged ion-pairing reagent such as naphthalene-2-sulphonate, NS^-, into an immiscible organic phase occurs as the ion-pair of the amine, HANS.

$$HA^+_{aq} + NS^-_{aq} \rightleftharpoons HANS_{org}$$

which is described by the extraction constant

$$K_{EX(HANS)} = [HANS]_{org} / [HA^+]_{aq}[NS^-]_{aq}$$

[ ] denotes molar concentrations.

The pH of the aqueous phase will influence the extraction of HANS due to

$$HA^+_{aq} + H_2O \rightleftharpoons A_{org} + H_3O^+ \quad \text{and}$$

$$NS_{org} + H_2O \rightleftharpoons NS^-_{aq} + H_3O^+$$

which shows that whilst an acidic pH is necessary for protonation and good aqueous solubility of the organic amine, at a sufficiently low pH the ionisation of NS^- will be suppressed and NS will partition into the organic phase. The optimum pH for minimum transfer of NS to the organic phase whilst providing maximum protonation of organic amines was therefore required to be determined experimentally.

Chloroform is suitable as the major component of the organic phase because it separates well from water due to its high density (1.47 gml^{-1}) and is therefore good for segmentation purposes and works well in a phase separator. Its solubilization ability is largely due to its proton donor property (119).

The addition of a proton acceptor solvent such as pentan-1-ol, which has very little solubility in water, considerably increases the solvating strength of the chloroform phase. This would increase the extraction efficiency of the organic phase for analyte-NS ion-pairs, but also for the NS reagent, which would raise the baseline absorbance. The influence of % v/v pentan-1-ol added to the chloroform was determined using the post-column system under constant chromatographic conditions. The absorbance of the organic phase due to the transfer of NS reagent was measured off-line using a UV-VIS spectrophotometer (Perkin-Elmer 550S), and is shown in Fig. 5.3.

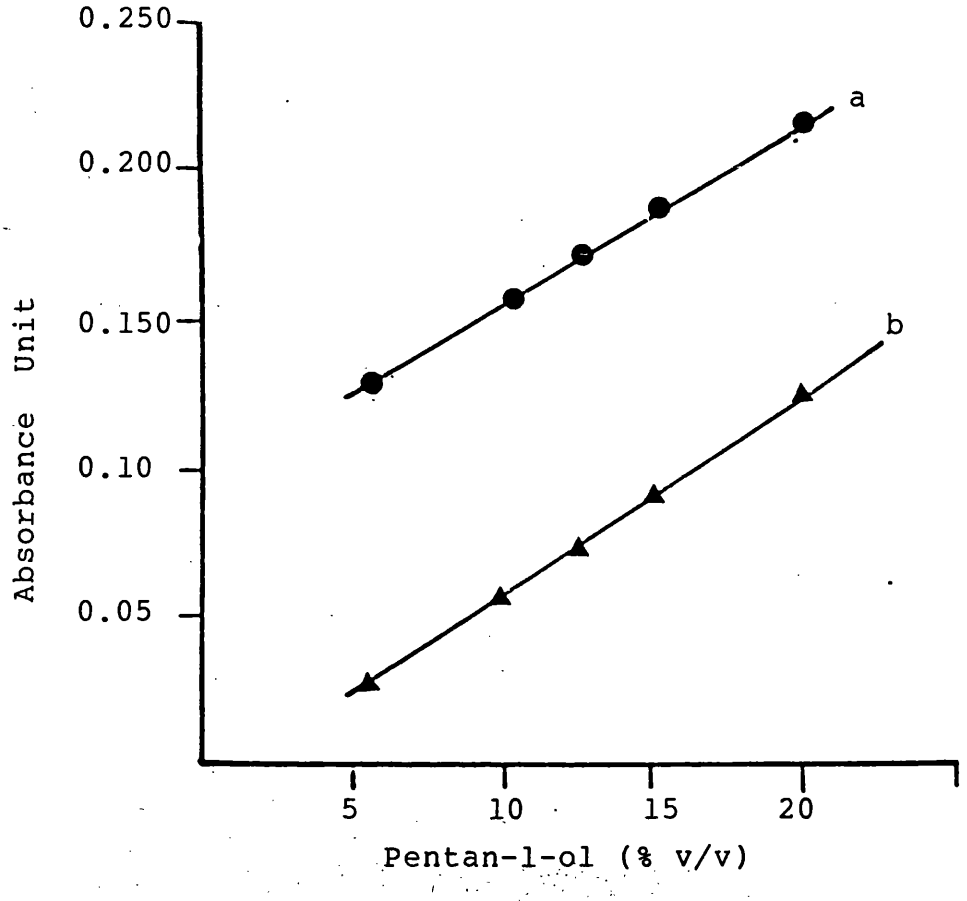


Figure 5.3. Influence of pentan-1-ol concentration (%, v/v) on NS transfer for organic phase. (a - 254 nm; b - 274 nm)

Conditions

1. Organic phase - chloroform + pentan-1-ol (variable % composition).
2. Aqueous phase - mobile phase + NS reagent. (pH 3.5).

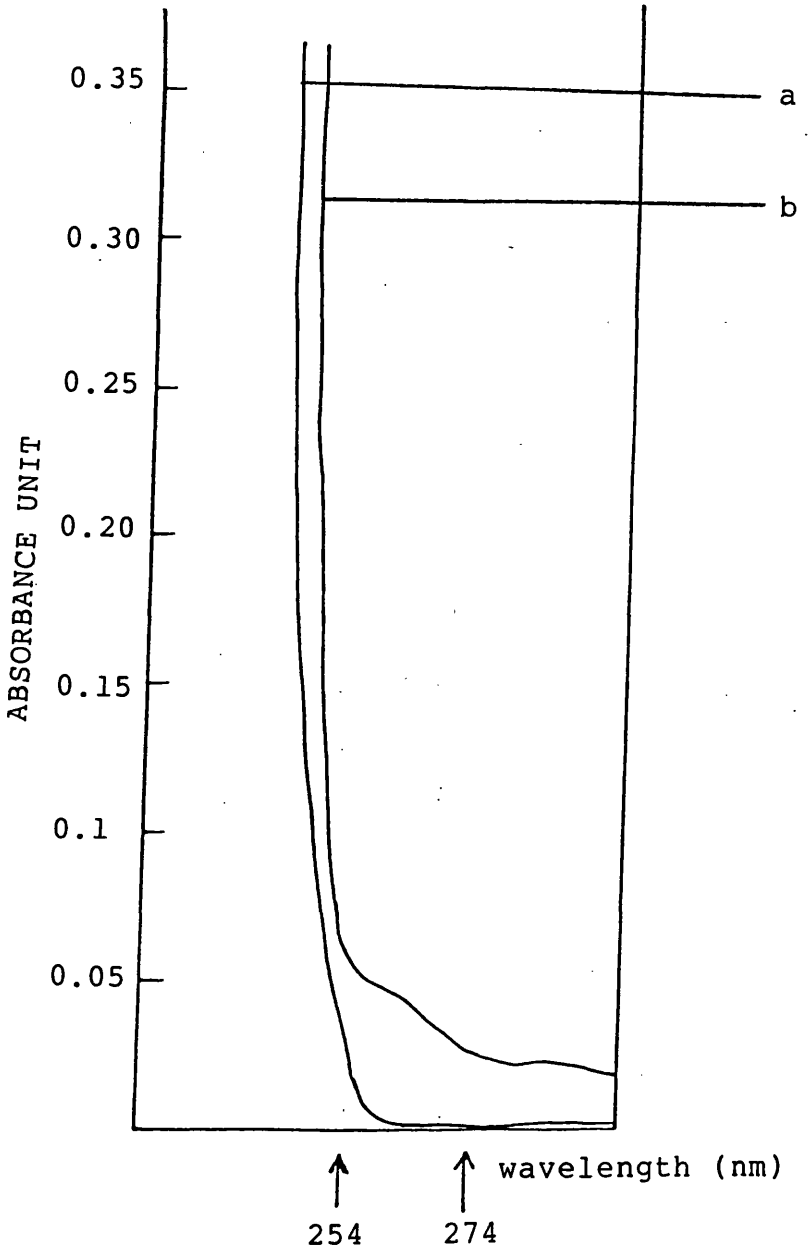


Figure 5.4. UV absorption spectrum of

a) 90% v/v chloroform + 10% v/v pentan-1-ol

b) 90% v/v chloroform + 10% v/v pentan-1-ol, equilibrated with chromatographic mobile phase + ion-pairing reagent (NS)

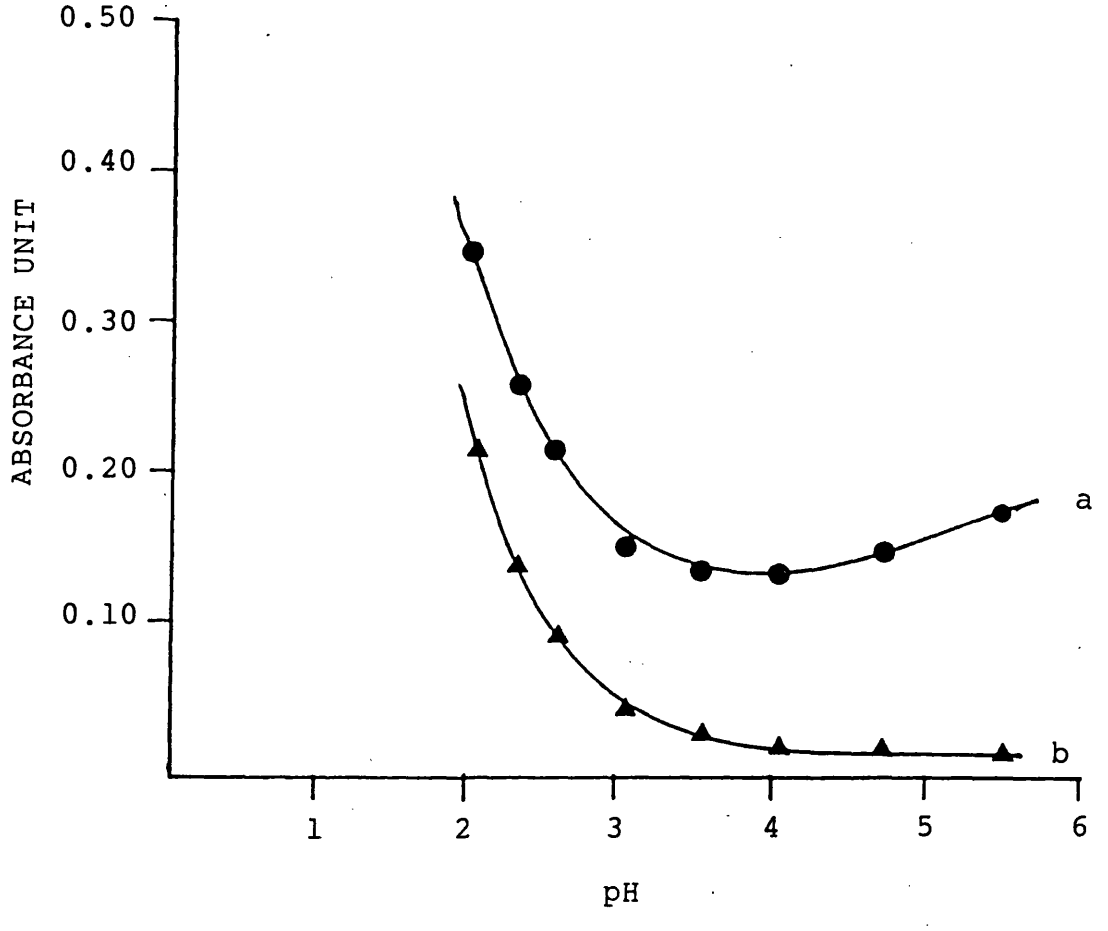


Figure 5.5. Influence of pH change on NS transfer to organic phase. (a = 254 nm; b = 274 nm)

Conditions

Organic phase - chloroform + pentan-1-ol (95% v/v + 5% v/v)

Aqueous phase - mobile phase + NS reagent (variable pH).

Absorbance was found to be linearly related to % v/v pentan-1-ol present, at both wavelengths used. The greater absorbance at 254 nm is mainly due to the greater absorbance of chloroform and pentan-1-ol at that wavelength compared to 274 nm, Fig. 5.4. These results indicated that for minimum background noise, it was preferable to restrict the % v/v pentan-1-ol to 5-10% and monitor at 274 nm.

The optimum pH range for the minimum transfer of NS reagent to the organic phase was then determined by maintaining the % pentan-1-ol in the organic phase to 5%, by keeping the chromatographic conditions constant but varying the pH of the NS reagent added post-column. Fig. 5.5 shows that at 274 nm the minimum pH is about 3.0, with little change in absorbance noted up to pH 5.5. The absorbance at 254 nm was at least three times greater, with a minimum at pH 3.5.

5.4 QUANTITATIVE STUDIES USING HPLC WITH POST-COLUMN EXTRACTION DETECTION

As a result of the preliminary studies to optimise the performance of the components of the post-column system, the following conditions were selected for the preparation of calibration curves for some of the Group A compounds.

a) Chromatography

Column: Hypersil-SAS, and Nucleosil-SA were both

studied.

Mobile phase: 0.02 M phosphate buffer (pH 5.4) - methanol - acetonitrile (90:5:5; % v/v/v)

b) Ion-pair reagent

0.01 M Na naphthalene-2-sulphonate in 2.0 pH.

c) Reactor

20 x 2 mm I.D. column packed with 150-200 μm ballotini beads.

d) Organic phase

Chloroform-pentan-1-ol (90:10 % v/v) equilibrated with aqueous phase (mobile phase + NS reagent).

e) Segmentor

Tee piece, PTFE, 0.8 mm I.D.

f) Extraction

PTFE tubing, 3 metre x 0.8 mm I.D. 6.7 cm coil diameter.

g) Detection

UV at 274 nm, 0.02 AUFS.

5.4.1 Nucleosil SA

Figure 5.6 shows a typical chromatogram for the resolution of seven Group A compounds obtained using the post-column system. It was found that p-hydroxy-norephedrine, p-hydroxyamphetamine, and p-hydroxy-N-methylamphetamine were poorly detected because of their strongly polar functional groups and acidic nature of the reagent-eluent aqueous phase (pH 3.0-3.5) reduced

Figure 5.6.

Post-column conditions for the calibration of some Group A compounds.

Aqueous phase

a) stationary phase: Nucleosil SA 5 m 50 mm x 4.6 mm
mobile phase: 82.5% (v/v 0.2 M KH_2PO_4 (pH 5.0)
10% v/v methanol + 7.5% v/v acetonitrile.
Temperature: 57°C*
Flow rate: 1 ml/min
Injection volume: 10 µl

b)0.01M Sodium naphthalene-2-sulphonate
Flow rate: 0.30 ml/min

Organic phase
90% v/v chloroform + 10% v/v pentanol
Flow rate: 2.6 ml/min
λ_{max}: 274 nm

* Aqueous phase was always cooled down from 57°C with ice crystals when using Nucleosil SA column.

Legend

1	p-Hydroxynorephedrine
2	p-Hydroxyamphetamine
3	p-Hydroxymethylamphetamine
5	Dexamphetamine
6	Phendimetrazine
7	Methylamphetamine
9	Mephentermine

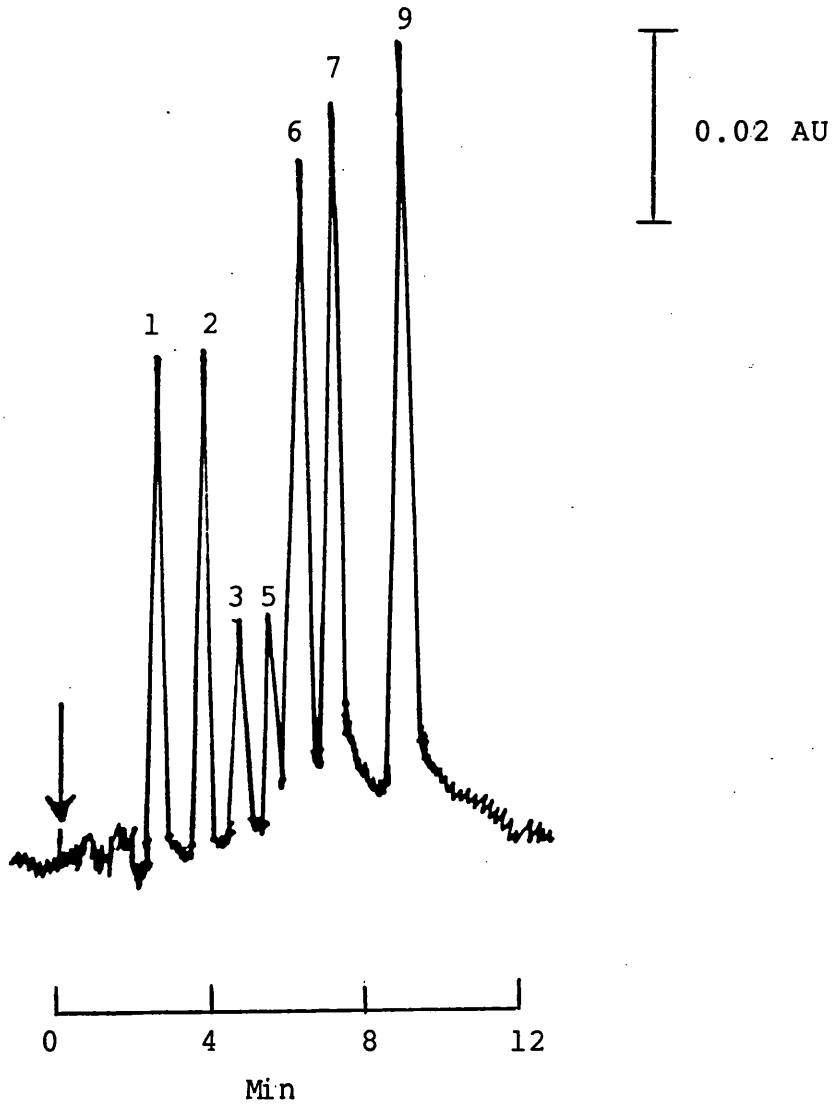


Figure 5.6. Typical post-column separation of some Group A compounds, variable concentrations. Conditions as on opposite page.

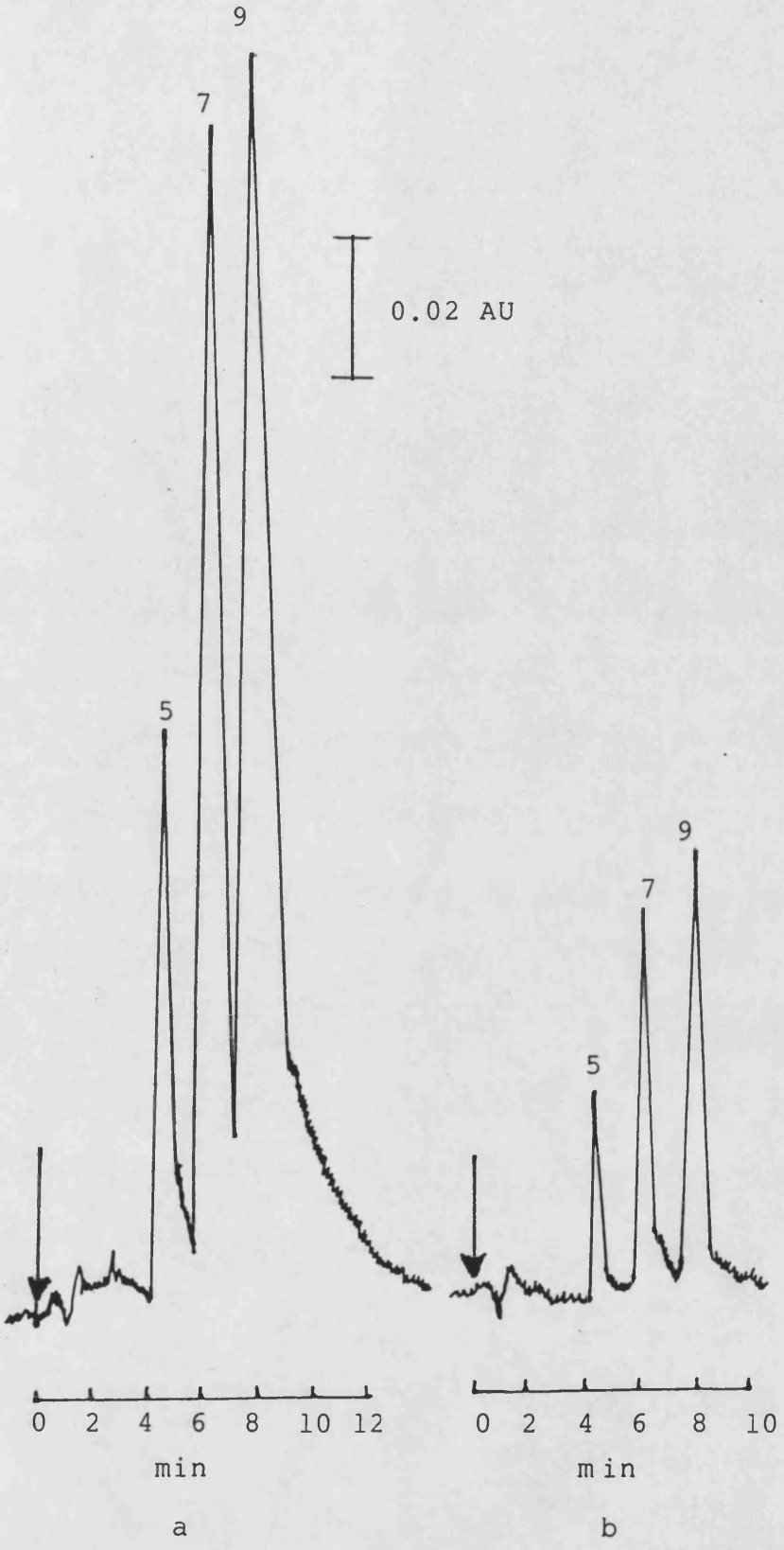


Figure 5.7 Highest (a) and lowest (b) concentrations for the Post-column calibration of dexamphetamine (5), methylamphetamine (7) and mephentermine (8). Conditions as in Figure 5.6.

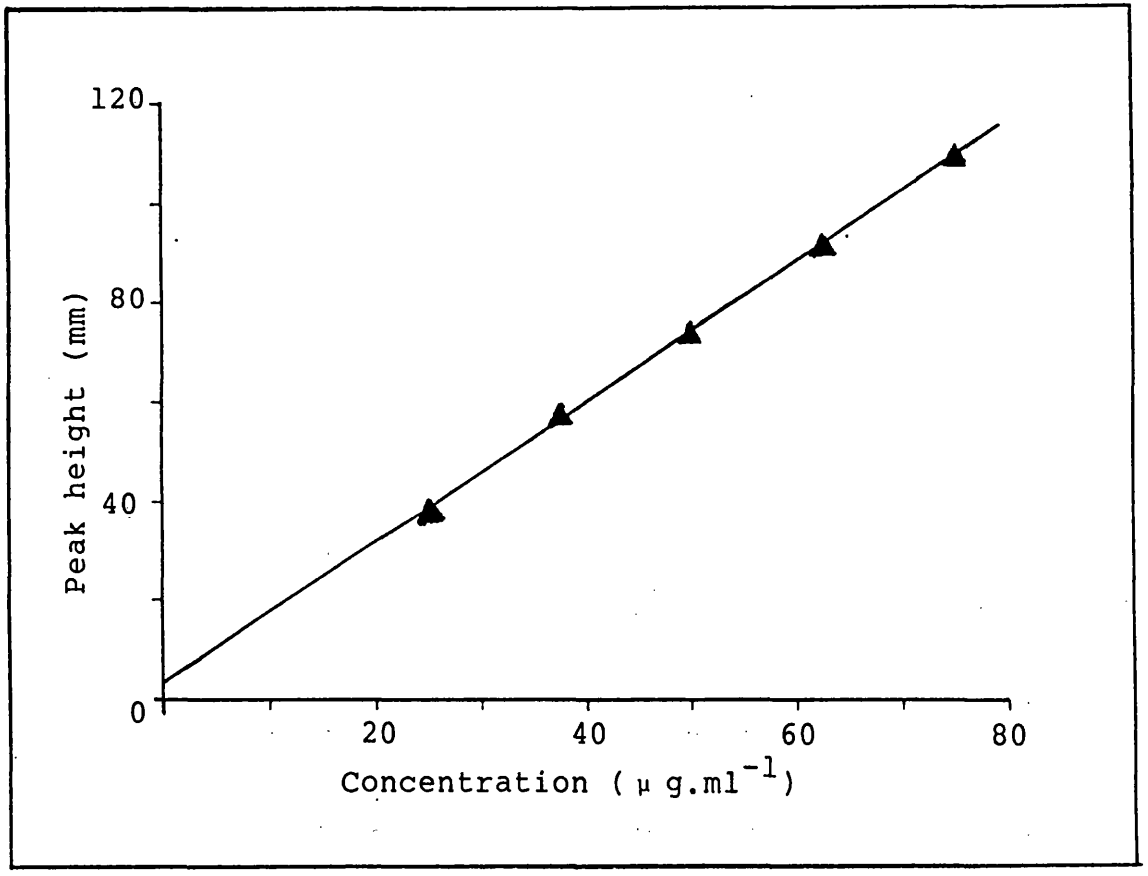


Figure 5.8 Five-point calibration of Dexamphetamine (5).
Conditions as in Figure 5.6.

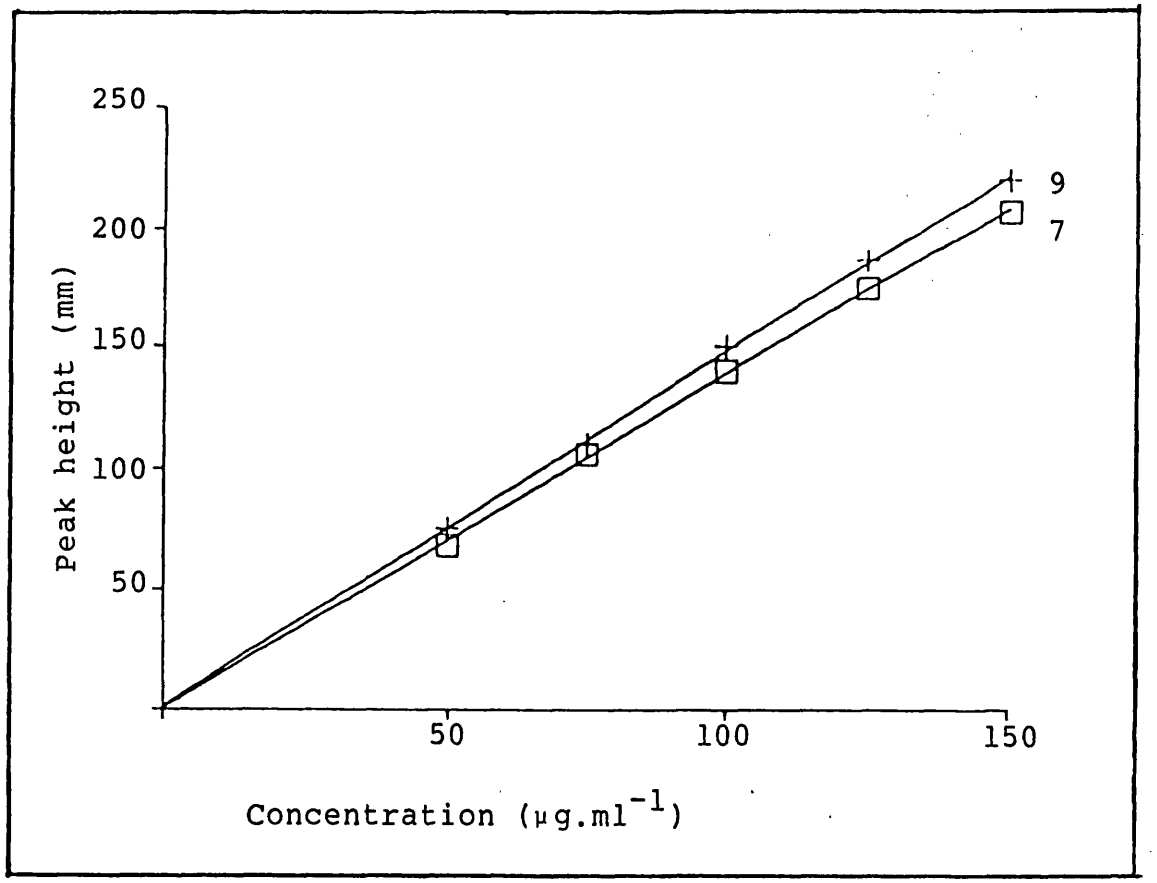


Figure 5.9 Calibration of methylamphetamine (7), mephentermine (9). Conditions as in Figure 5.6.

the extraction of the ion-pair into the chloroform layer, and so these compounds were not studied quantitatively.

Five calibration solutions were made for a mixture containing dexamphetamine (2.5-7.5 μg ml^{-1}) methyl-amphetamine (10-30 μg ml^{-1}) and mephentermine (10-30 μg ml^{-1}), and Fig. 5.7 shows the chromatograms for the highest and lowest concentrations in this mixture. Linear calibrations were obtained for each compound (Fig. 5.8, 5.9) with correlation coefficients > 0.999 and relative standard deviations (n = 10) < ± 1.52%, even though no internal standard was used (Table 5.2).

Table 5.2. Statistical analysis of the results for the calibration of dexamphetamine (5), methyl amphetamine (7) and mephentermine (8). Conditions as in Figure 5.1.

Compounds	DEXA (5)	METH (7)	MEPH (9)
Correlation Coefficient	0.9993	0.9993	0.9991
Slope	1.424	1.384	1.464
Intercept	3.2	0.60	1.80
Std. deviation of slope	0.0244	0.2117	0.0222
Std. deviation of intercept	1.2961	2.245	2.3495
% RSD (n = 10)	1.51	1.52	1.46

Hypersil SAS

Figure 5.10 is a typical chromatogram of the separation of seven Group A compounds. The three p-hydroxy-metabolites were not included here because of the reasons given under nucleosil. Figures 5.11(a) and 5.11(b) are the chromatograms of the highest and lowest concentrations for calibration while Figures 5.12-5.14 are 5-point calibration plots for a mixture containing 2-phenylethylamine (4) (15-60 $\mu g ml^{-1}$), dexamphetamine (5) (15-60 $\mu g ml^{-1}$), phenmetrazine (6) (25-100 $\mu g ml^{-1}$), methylamphetamine (7) (33-120 $\mu g ml^{-1}$), phenedimetrazine (9) (25-100 $\mu g\ ml^{-1}$), mephentermine (8) (33-120 $\mu g ml^{-1}$) and chlorphentermine (10) (25-100 $\mu g ml^{-1}$). Linear calibrations were also obtained for each compound with correlation coefficients > 0.99, except for phenmetrazine which is 0.943, intercepts were small and relative standard deviations ($n = 10$) $< \pm 1.7\%$ without the use of an internal standard (Table 5.3).

Figure 5.10.

Post-column conditions for the calibration of some Group A compounds.

Aqueous phase

a) stationary phase: Hypersil SAS 100 x 2.1 mm
   mobile phase: 90 % v/v 0.02 M phosphate buffer (pH 5.4)
   5 % v/v methanol + 5 % v/v acetonitrile.
   Temperature: 35°C
   Flow rate: 0.5 ml/min
   Injection volume: 10 µl

b) 0.01M Sodium naphthalene-2-sulphonate
   Flow rate: 0.15 ml/min

Organic phase

90% v/v chloroform + 10% v/v pentanol
Flow rate: 1.30 ml/min
λ_{max}: 274 nm

Legend

4	2-Phenylethylamine
5	Dexamphetamine
6	Phendimetrazine
7	Methylamphetamine
8	Mephentermine
9	Phenmetrazine
10	Chlorphentermine

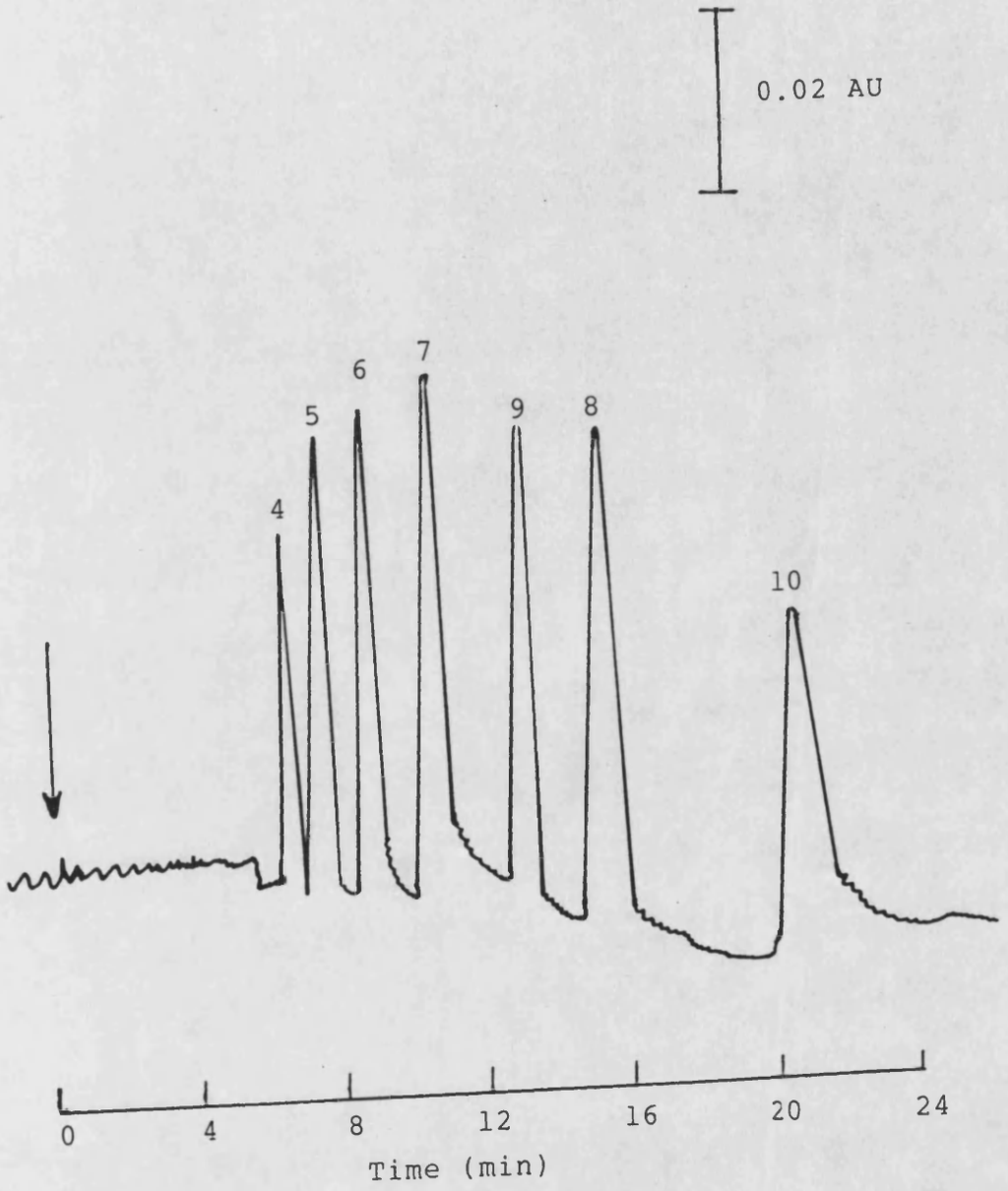


Figure 5.10 Typical post-column separation of some Group A compounds. Conditions as on opposite page.

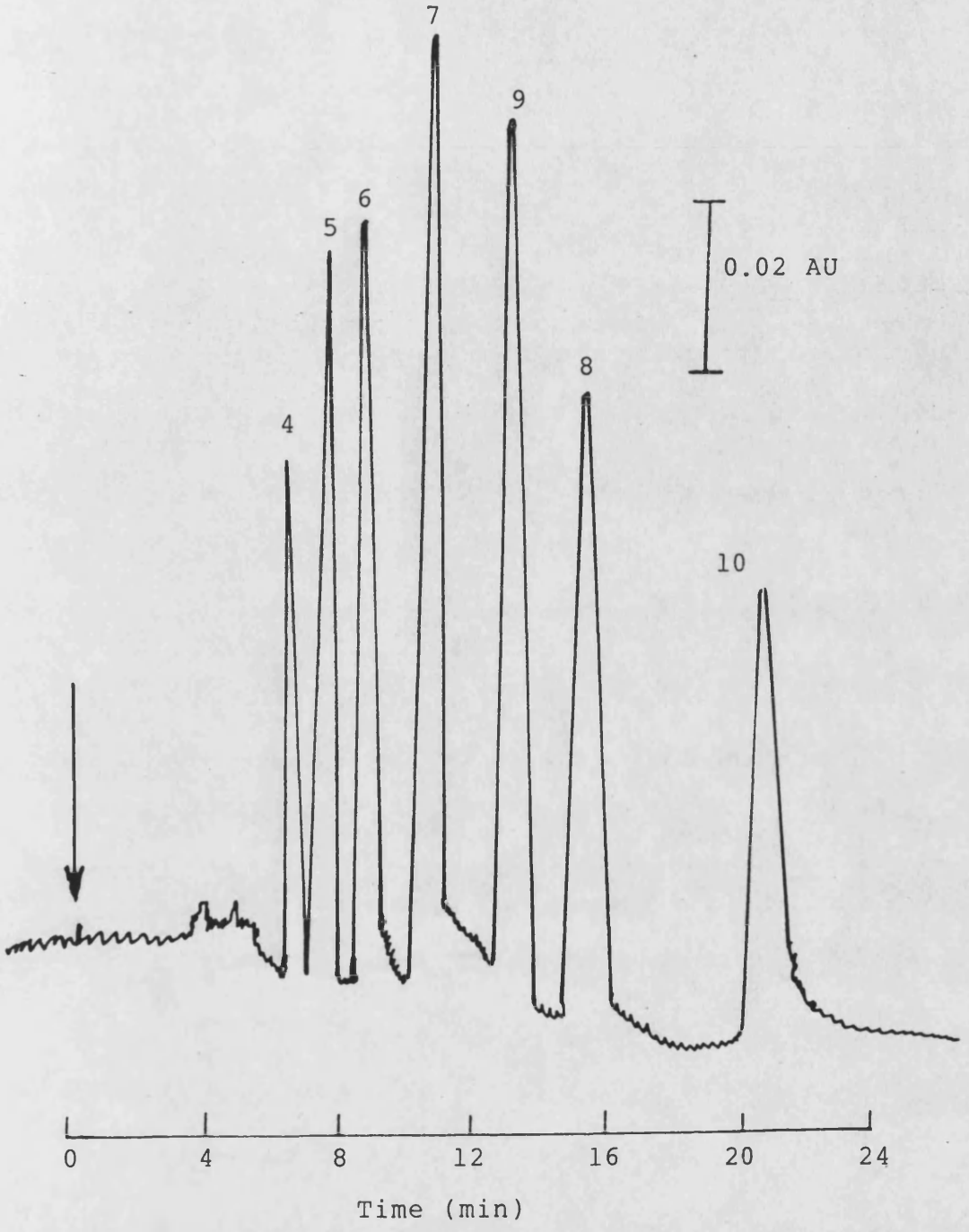


Figure 5.11a Highest concentration for the post-column calibration of some Group A compounds. Compounds and conditions as in Figure 5.10.

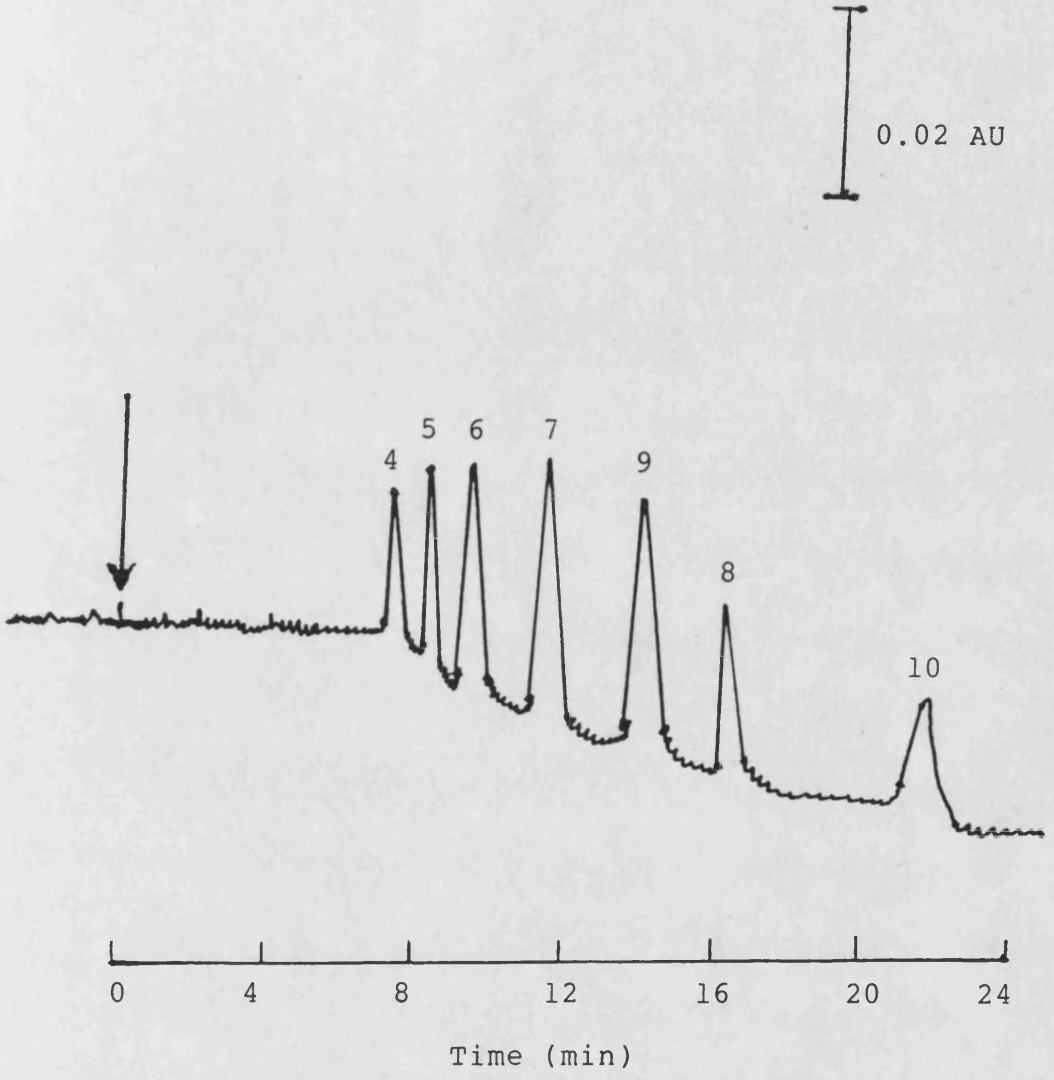


Figure 5.11b Lowest concentration for the post-column calibration of some Group A compounds. Compounds and conditions as in Figure 5.10.

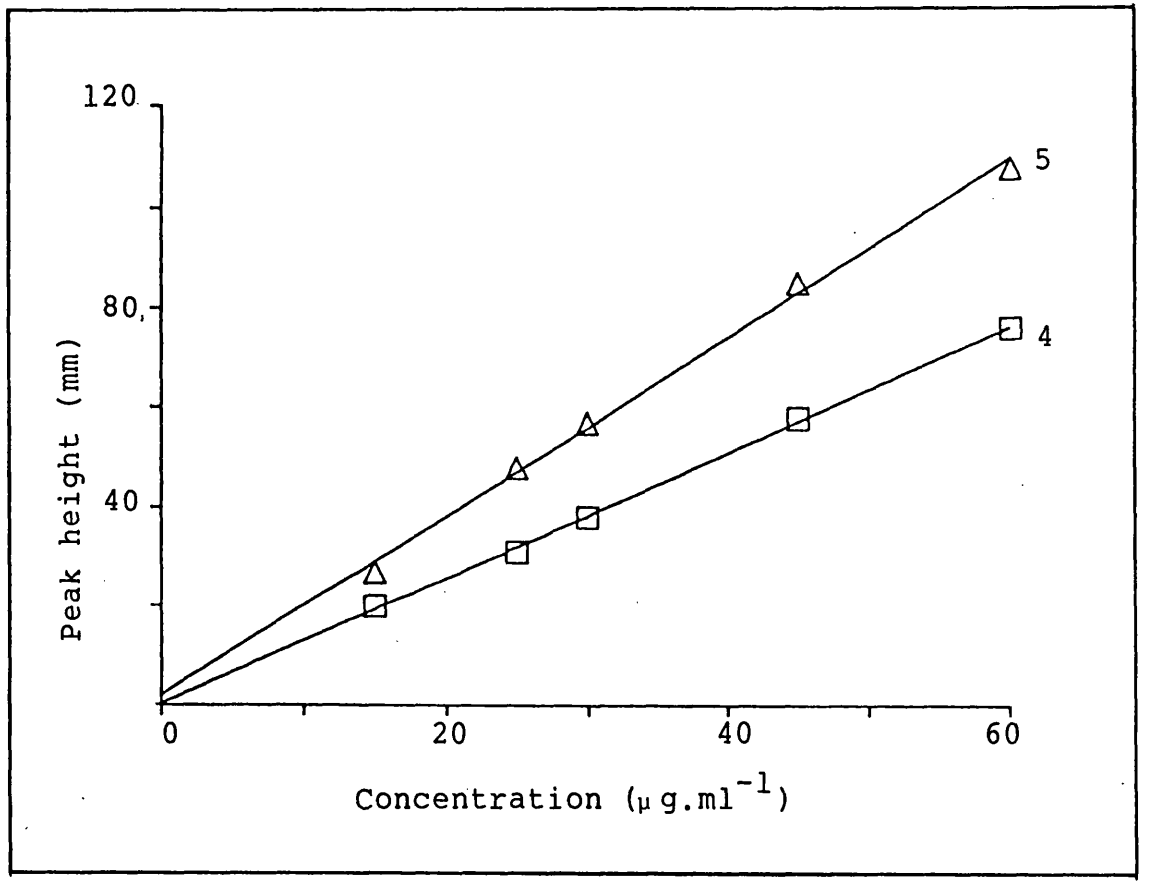


Figure 5.12. Calibrations of 2-phenylethylamine (3) and dexamphetamine (5)

Conditions as in Figure 5.10.

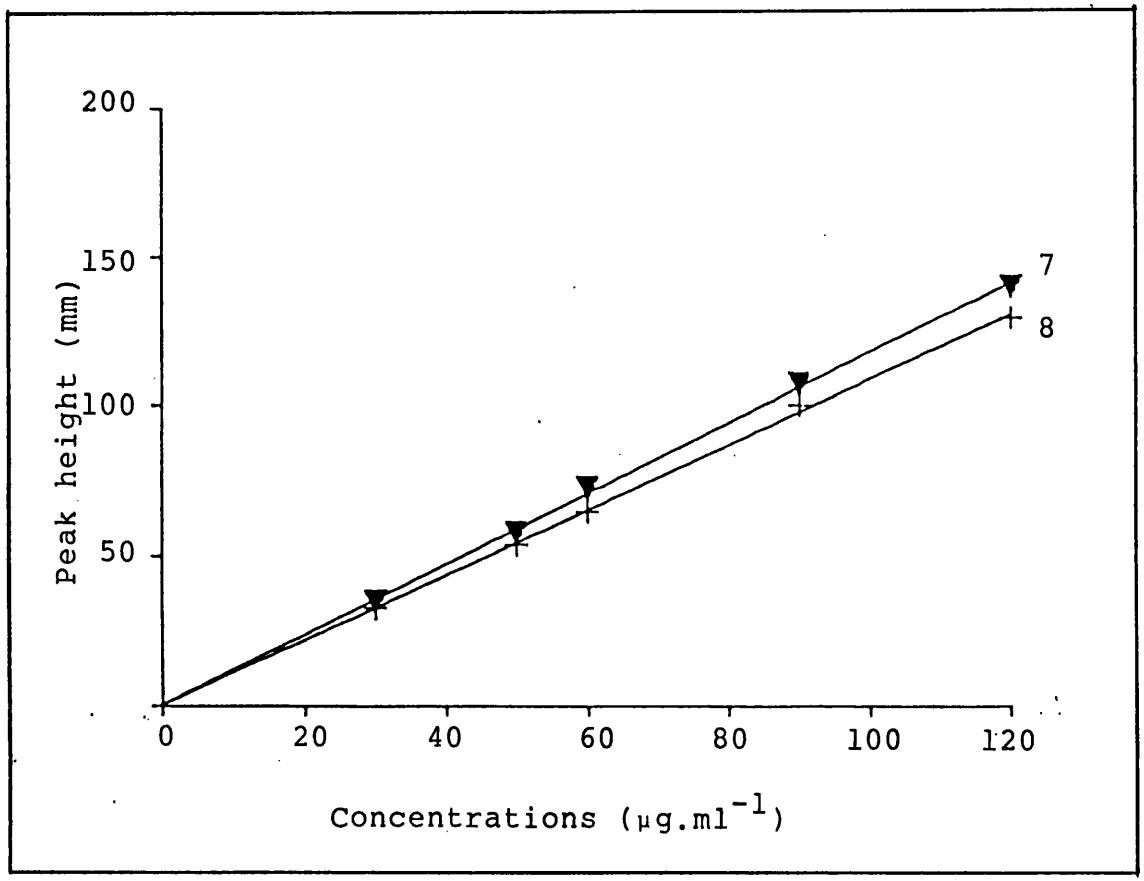


Figure 5.13 Calibrations of methylamphetamine (7) and phendimetramine (8). Conditions as in Figure 5.10.

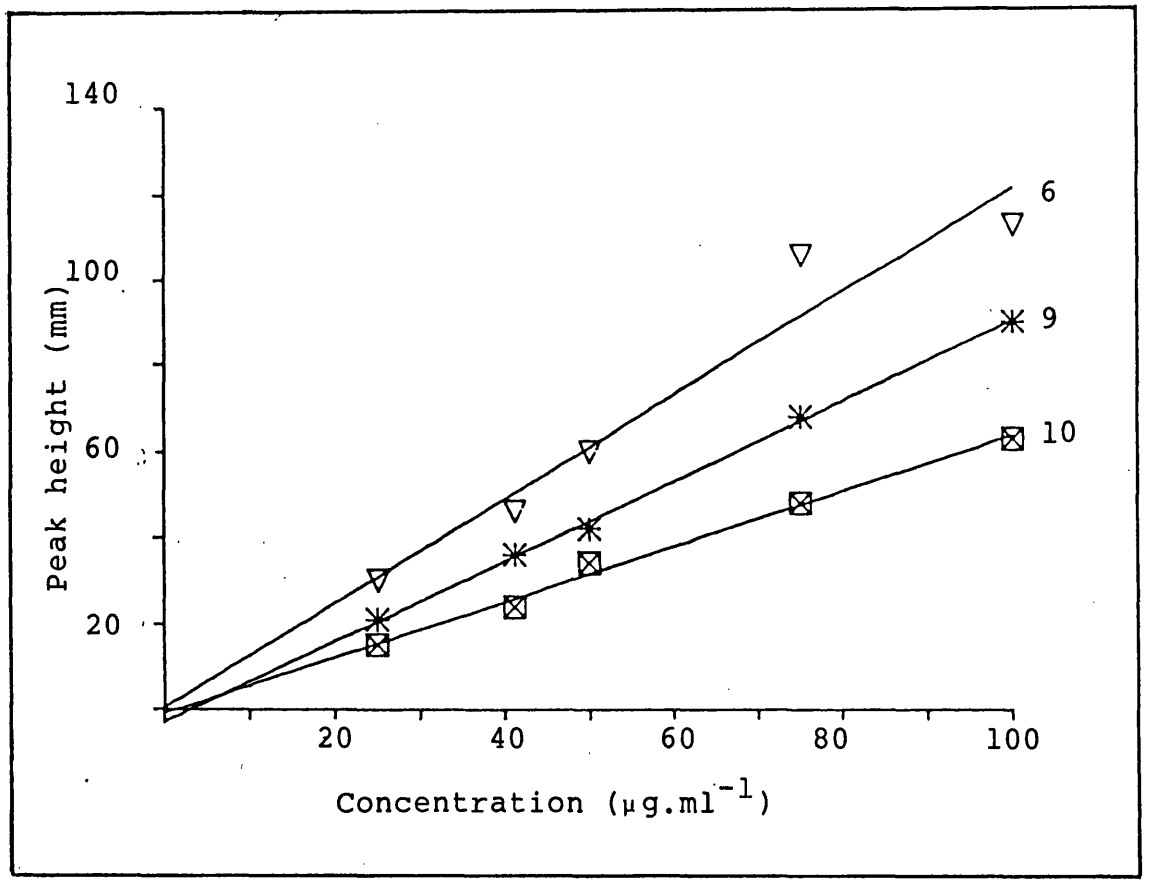


Figure 5.14 Calibrations of phenmetrazine (6), phendimetrazine (9) chlorphenmetrazine (10). Conditions as in Figure 5.10.

Table 5.3. Statistical analysis of the calibration of 2-phenylethylamine (4), dexamphetamine (5), phenmetrazine (6), methylamphetamine (7), phendimetrazine (9), mephentermine (8) and chlorphentermine (10). Conditions as in Figure 5.11.

Compounds	3	5	6	7	8	9	10
Correlation Coefficient	0.9990	0.9965	0.9425	0.9987	0.9988	0.9985	0.9927
Slope	1.264	1.796	1.210	1.17	1.08	0.9295	0.6447
Intercept	0.36	2.14	0.486	0.70	0.60	-2.754	-0.758
Std. dev. of slope	0.0236	0.0611	0.1726	0.0241	0.0213	0.0207	0.03193
Std. dev. of Intercept	0.9045	2.345	11.039	1.8499	1.6354	1.3249	2.0421
% RSD (n=10)	1.32	1.58	2.95	1.53	1.49	1.53	1.61

SECTION 6

CONCLUSION

SECTION 6. CONCLUSIONS

Numerous workers in the last few years have strived to improve both methodology and sensitivity in the analysis of compounds of forensic interest. In 1973 Cashman et al. (121) used long stainless steel columns (1500 x 2 mm ID) packed with pellicular silica material (Corasil II) to separate phenethylamines and compared the results with that of an ion-exchange material (DA-X4) packed in glass columns (250 x 6 mm i.d.). They found that the ion-exchange material gave better separations at a pH of 9.0 and at 64°C. However peaks were broad and sensitivities not good due to low column efficiencies.

With the introduction of fully porous micro-particulate silica (10 μm), column efficiencies improved dramatically, which was further improved when 5 μm material became readily available. In 1975 Jane (122) described the use of 6 μm Partisil silica as 250 x 4.6 mm I.D. columns to separate a wide range of drugs of abuse using methanol-2N ammonia solution-1N ammonium nitrate solution (27: 2: 1, % v/v) and UV detection at 254 nm. It was particularly suitable for the identification and quantitation of mixtures of basic drugs because peak shapes were good and most analyses required 15 mins or less. Sensitivity limits were dependent upon the molar absorptivity of each compound

at 254 nm. Achari et al. in 1977 (123) also used silica (Partisil 10 μm) with methanol-dichloromethane (3:1, v/v) containing 1% v/v ammonium hydroxide (29% NH_3) for pharmaceutical dosage forms of 29 basic drugs, and found the columns did not deteriorate due to the alkali, probably due to the low water content. The importance of the brand of silica chosen for this type of system was demonstrated in 1984 by Law, Gill and Moffat (124) who compared the retentions of a 7-component test mixture on Hypersil, Spherisorb S5W, Nucleosil 50-5 and Zorbax BP-Sil. Although no alterations in retention order occurred, total analysis times varied from 4 to 6 minutes using a mobile phase of methanol-aqueous ammonium nitrate buffer, pH 10.1 (9:1 % v/v). Spherisorb S5W was selected to examine the retention behaviour of 84 basic drugs of forensic interest. Although peak shapes were good, k' values were in a small range, for example for 36 amphetamine-related compounds k' = 0.14 to 2.48. Whilst this provided short analysis times it also meant that the system was not very selective, having limited discriminatory power. The retention mechanism of this chromatographic system was thoroughly investigated by Law in 1987 (125) who concluded that it was mainly a cation-exchange mechanism influenced by solute pKa (in the range 8-11) and the stereochemistry and degree of substitution at or near the basic centre. Because

Spherisorb S5W had been chosen as the standard silica material for use throughout the Forensic Science Service Laboratories in the U.K., this material was used by Jane et al. in 1985 (126) for a very comprehensive study using 462 basic drugs. Their retentions were determined using methanolic ammonium perchlorate (10 mM) plus 1 ml/l methanolic sodium hydroxide (0.1 M) pH 6.7, and their relative detection responses by UV at 254 nm and electrochemical at +1.2 V compared. The relative response ratio was found to cover a wide range that could be used to considerably improve the possible identification of analytes. This was necessary because only 15 analytes had $k' > 6.0$. Electrochemical detection was much more sensitive than UV at 254 nm, but limits of detection were not investigated. For aliphatic amines, the ease of oxidation is tertiary > secondary > primary so that for the compounds studied in this thesis, EC detection would have the disadvantage of having a wide range of sensitivity limits. The authors showed that this chromatographic system could be used with post-column derivatisation using fluorescence with 2-mercapto-ethanol-O-phthalaldehyde for primary amines such as amphetamine, with a sensitivity limit probably about 20 ng on-columm. UV at 215 nm and 0.03 AUFS was sensitive down to about 25 ng of amphetamine. The authors suggested that 205 nm could be used but did not

use this wavelength. Since methanol (Fisons, HPLC grade) has an absorbance of 0.4 at 210 nm, it is unlikely that the solvent systems used by the silica column methods described above would be able to improve upon that achieved at 215 nm.

Reversed-phase column materials have been less successful for basic compounds due to excessive retention caused by residual silanol groups on the bonded silica surface. Twitchett and Moffat (127) found μ-Bondapak-C_{18} to be suitable for acidic and neutral compounds but not basic compounds of forensic interest. Although Baker *et al*. (128) obtained better column efficiencies using the same packing material, selectivity was very limited, and they proposed using the ratio of analyte absorbances at 254 nm and 280 nm in order to assist peak identification.

Cation exchange chromatography was examined by Twitchett *et al*. (129) for 30 compounds of widely differing structures and found to be useful for basic substances although column longevity was questionable.

In all the above cases, high carbon loading materials coupled with high percentage of organic eluents have been used, and selectivities are also low. In this thesis, two approaches to detecting these compounds of abuse have been developed:

i) HPLC using columns with low carbon loadings, monitored at 205 nm. In this approach short (50 mm and

100 mm) and narrow bore (2 mm) analytical columns have been found to provide selective and sensitive detection for all the compounds studied. Analytical times are easily adjusted by alteration of the percentage of organic modifiers.

ii) Post-Colum Derivatization. The low percentage of organic modifiers (5% methanol + 5% acetonitrile) in the mobile phase developed for Group A compounds meant that this chromatographic system could be used with a post-column extraction detection method. This approach using sodium naphthalene-2-sulphonate, and described in Section 5, was found to be more complex to assemble and operate, but linear calibrations were obtained even though detection was not enhanced as anticipated.

It is appropriate to compare the results obtained using the two approaches.

6.1 Assessment of Chromatographic Peaks

It is highly desirable that analyte peaks should be tall, narrow and symmetrical in order to maximise sensitivity and range of linearity in calibration curves. This can be more readily achieved when chromatographic conditions are chosen to produce predominantly low k' values. However the objective of this study was to develop systems with good selectivity, which meant that the k' range must be large enough to accommodate all the analytes on the

chromatogram. This was achieved with Hypersil-CN, Hypersil-SAS and Nucleosil SA, as described in Section 3. Hypersil-SAS and Nucleosil SA were also both employed using identical chromatographic conditions but with post-column ion-pair extraction detection as described in Section 5, and so the chromatograms of these two procedures may be compared directly. Table 6.1 summarises the relevant data.

6.1.1 Peak Widths (W)

These were measured at 10% peak height and converted into seconds. For the Nucleosil SA system used at 205 nm the peak widths were between 18 and 48 secs for k' values between 0.43 and 9.0. This is satisfactory considering the good resolutions obtained. When this system was used with the post-column detector conditions as in Fig. 5.1 and 5.2, peak widths were increased by 6 to 8 secs, representing a 15% increase. Considering the complexity of this system, this is also very satisfactory.

The Hypersil SAS system gave excellent selectivity but required a wider range of k' values, leading to increased peak widths, 18 to 84 secs for k' values between 2.43 and 14.0. These widths were further increased by 12 to 18 secs when the post-column system was added under the conditions in Fig. 5.10, representing a 20-33% increase. This is less

HYPERSIL SAS

			Chromatography at 205 nm Condition as in Figures 3.23 and 3.32				Post-Column at 274 nm Conditions as in Figures 5.6 and 5.10					
		k'	t_R (min)	W (sec) at 10% of the base	As	M_2 statistical moment	t_R (min)	W (sec) at 10% of the base	As	M_2	Band broadening due to post-column sec	Band broadening due to post-column % of W
4	2-phenylethylamine	2.43	5.40	35.0	1.0	0.48	7.50	48.0	1.0	0.86	12.0	33.3
5	Dexamphetamine	4.0	6.20	42.0	1.1	0.69	8.10	54.0	1.15	1.16	12.0	28.6
6	Phemetrazine	4.5	7.70	48.0	1.13	0.91	9.60	60.0	1.3	1.52	12.0	25
7	Methylamphetamine	5.43	9.50	60.0	1.50	1.64	11.60	72.0	1.9	2.71	12.0	20
8	Phendimetrazine	8.30	14.10	66.0	1.15	1.73	16.0	84.0	1.4	3.09	18.0	27
9	Mephentermine	7.14	12.0	60.0	1.40	1.58	14.0	72.0	1.8	2.64	12.0	20
10	Chlorphentermine	14.0	20.0	84.0	1.0	2.63	22.0	102.0	1.3	4.38	18.0	21

NUCLEOSIL SA

1	p-OH-norephedrine	0.43	1.2	18.0	1.0	0.12	2.70					
2	p-OH-amphetamine	1.6	1.8	18.0	1.0	0.12	3.10					
3	p-OH-methylamphetamine	2.3	2.3	24.0	1.0	0.21	3.58					
4	2-phenylethylamine	2.6	2.5	24.0	1.1	0.22	3.80					
5	Dexamphetamine	4.0	3.5	30.0	1.1	0.35	4.80	36.0	1.13	0.51	6.0	20
6	Phenmetrazine	5.14	4.3	42.0	1.12	0.69	5.60					
7	Methylamphetamine	6.43	5.2	42.0	1.8	0.90	6.4	48.0	1.25	0.95	6.0	14.3
8	Phendimetrazine	8.0	6.3	48.0	1.8	1.17	7.58					
9	Mephentermine	9.0	7.0	48.0	1.25	0.95	8.0	54.0	1.29	1.22	6.0	12.5

* Peak asymmetry has been calculated only for compounds calibrated with the post-column system.

satisfactory and is probably caused by the lower aqueous and organic flowrates employed here,compared to the Nucleosil SA system.

6.1.2 Peak Asymmetry (As)

Chromatographic systems for basic compounds frequently display asymmetrical peaks due to excessive interaction between acidic silanol groups and protonated amines. This can result in a substantial loss of sensitivity and resolution. Table 6.1 and Figs. 3.10 show that the three low carbon column materials used in this thesis gave peaks that for most analytes were satisfactory (As = 0.9 - 1.2). A few more basic analytes such as methylamphetamine, phendimtrazine, gave higher values (As = 1.5-1.8) but even these are not excessive. A possible explanation for the better performance of the cyanopropyl-, short alkyl- and ion-exchange materials compared to ODS materials is that the surface coverage of the low carbon materials is much higher so that fewer silanols remain to cause analyte tailing. A direct comparison of the peaks generated by the post-column system with those generated at 205 nm, Table 6.1, show only a slight increase in asymmetry (As = 1.0-1.9). This is surprising considering the two 'tee' pieces, 3 metres of tubing and phase separator between the analytical column and detector cell, and clearly illustrates the

effectiveness of segmented flow in maintaining analyte band integrity.

6.2 Analysis Time

The analysis times required for the three column systems developed here are guidelines only and are capable of considerable modification according to the range of analytes chosen and mobile phase composition selected. Column efficiency is also an important factor which influences the mobile phase flowrate. However, the three systems all produced chromatograms with the analytes regularly eluted, with little time wasted. It is felt that the cyanopropyl column in particular would be very suitable as a more selective alternative to the silica-methanolic ammonium nitrate system currently used to identify basic drugs.

The post-column system increased the analytical time by 1 minute for the Nucleosil SA column (total flowrate 4 ml min^{-1}) and by 2 minutes for the Hypersil SAS system (total flowrate 2 ml min^{-1}), Table 6.1. Considering the complexity of the system, this is a very small increase.

6.3 Sensitivity and Ease of use

A comparison of the sensitivity of detection at 205 nm compared with post-column detection at 274 nm, in Table 6.2 for Group A compounds, shows that the

Table 6.2.

Compound	log P	Minimum level of Detection 205 nm (ng)	Post-column 274 nm (ng)
p-OH-norephedrine	0.18	2	250
p-OH-amphetamine	0.20	2	250
p-OH-N-methylamphetamine	0.22	2	250
2-phenylethylamine	0.22	2	20
dexamphetamine	0.63	2.5	25
methylamphetamine	0.82	3.0	100
chlorphentermine	1.10	4.0	-
phenmetrazine	1.02	2.5	50
phendimetrazine	2.06	3.5	50

former is much more sensitive. For analytes that are very polar, such as the hydroxylated metabolites, extraction into the chloroform-pentan-1-ol phase, even as NS ion-pairs, was inadequate. The addition of a p-hydroxyl group to dexamphetamine raised its detection limit from 25 to 250 ng on-column. Whilst the extraction could probably be improved by making the organic phase more polar, it is unlikely that dramatic improvements would be obtained.

For the other analytes in Table 6.2 that are less polar (log P range 0.18 to 2.06) the levels of detection using the post-column system are 10 to 20 times higher than for the 205 nm system. Fig. 6.1 illustrates the enormous increase in sensitivity at 205 nm compared with 254 nm for a mixture of p-hydroxynorephedrine, p-hydroxyamphetamine, dexamphetamine, methylamphetamine and mephentermine. Fig. 6.2 illustrates the differences betwen 205 nm detection and post-column detection at 274 nm for the same mixture of compounds. It clearly demonstrates the lack of sensitivity of the post-column system towards the two hydroxylated metabolites.

In view of the relative simplicity of the 205 nm system and its greater sensitivity, this method is preferred. The cyanopropyl- column is recommended because of the shorter retention times and because no problems were encountered with column stability. No

Figure 6.1 Chromatograms of separation of some Group A compounds of variable concentrations. UV detection at:

a) 205 nm

b) 254 nm

Other conditions as in Section 3.3.

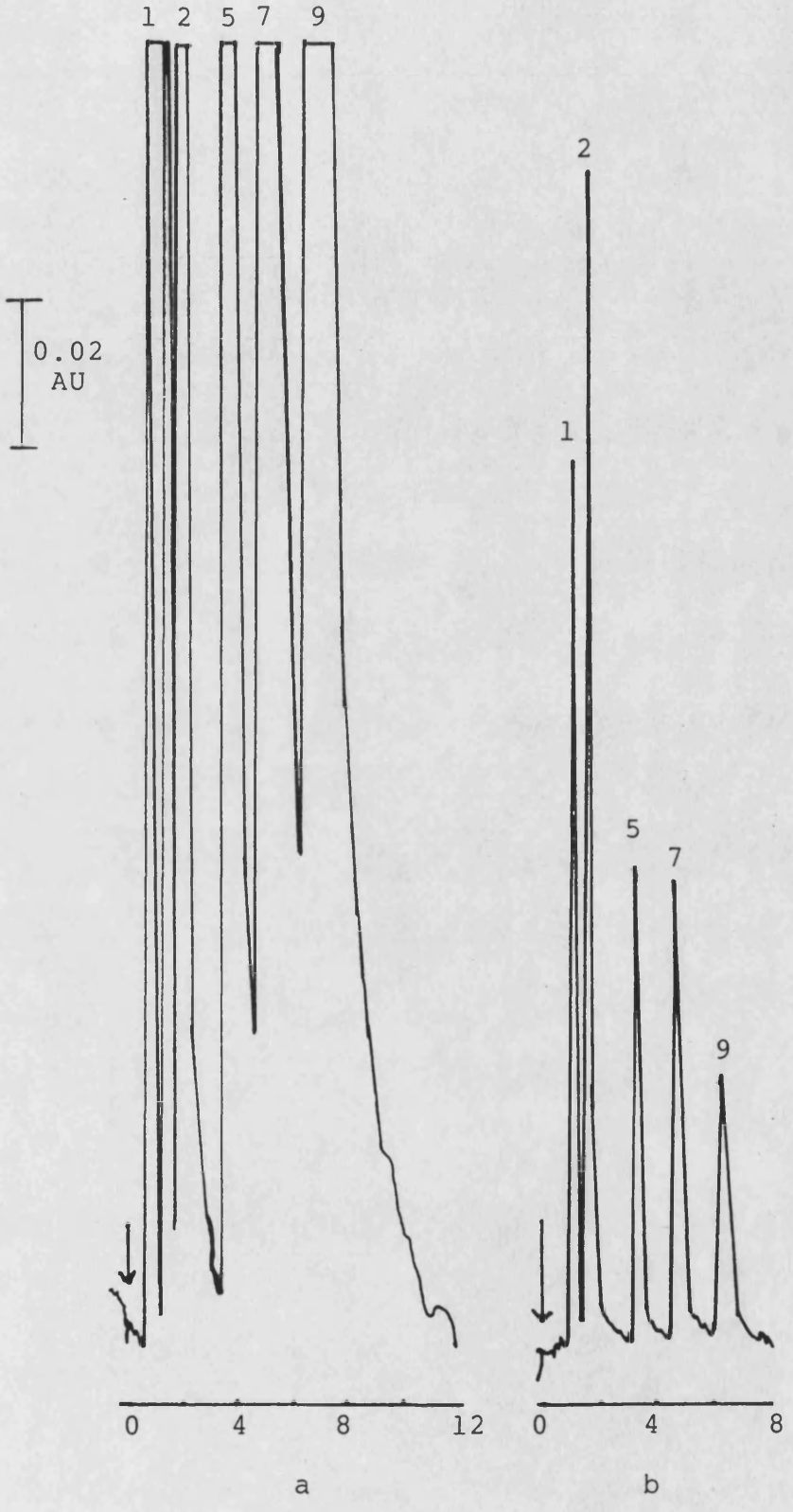


Figure 6.1.

Figure 6.2 Chromatograms of separation of some Group A compounds (variable concentrations).

a) Chromatography UV detection at 205 nm conditions as in Fig. 6.1.

b) Post-column UV detection at 274 nm conditions as in Fig. 5.6.

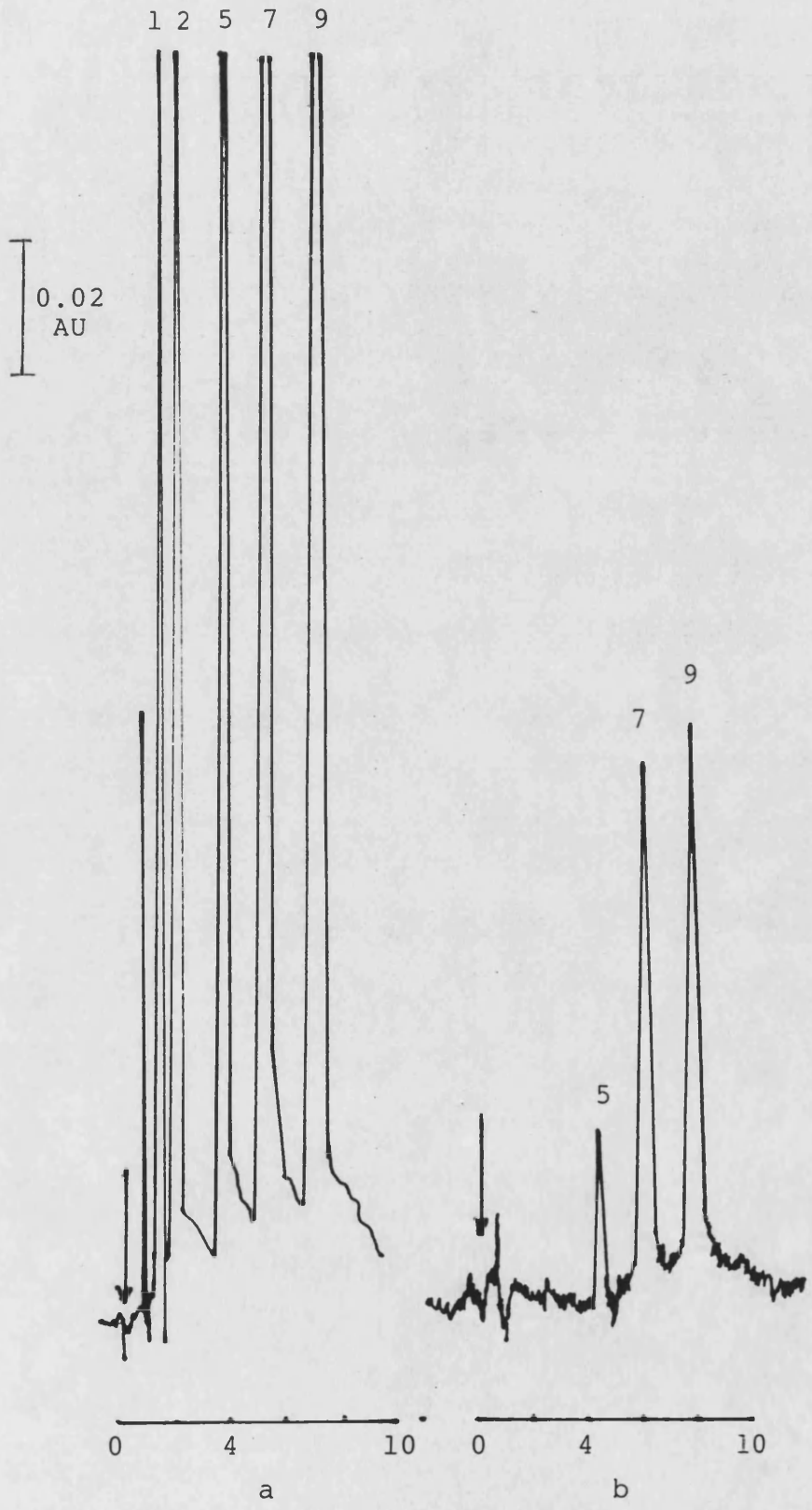


Figure 6.2

differences were noted between Hypersil-CPS and Spherisorb-Cyano materials. This method relies upon using good quality organic solvents and buffer salts to ensure a minimum UV background absorbance, and the use of a UV lamp with a good output at 205 nm. This output decreases as the lamp ages, and needs to be monitored regularly.

The post-column extraction detection system gave good linear calibrations but was more complex to use and adjust. In view of this and the sensitivity levels achieved, this method should be regarded as potentially useful but still in an early stage of development.

Suggestions for Further Work

1. The cyanopropyl- column could be used with the mobile phase developed for Group B compounds as a more sensitive and selective replacement for the silica column - methanolic ammonium nitrate system. The wider range of k' values obtained, plus dual UV detection at 205 nm and, say 254 nm would provide a better basis for peak identification.

2. Further work should be carried out on the post-column system to improve sensitivity levels. Different ion-pair reagents, including 9,10-dimethyoxyanthracene sulphonate for fluorescence detection, should enable much lower limits of detection to be obtained. The phase separator requires modification to permit its use without solvent leakage occurring.

3. It would be useful to make a systematic study of the effect of methanol, acetonitrile and propan-2-ol on analyte pKa values, so that a figure similar to that obtained for the effect of temperature may be determined. The possibility of an additive effect between organic modifier and temperature should also be considered. It should be possible for a study of

this kind to produce useful practical guidelines for chromatographers.

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Figure 2.8.

Configuration of phase separator for monitoring the organic phase.

Flowpath of organic phase:

1. Aqueous/organic segmented flow inlet
2. Separated organic phase plus some aqueous droplets
3. Re-entry into second separating channel
4. Exit of clean, organic phase to detector

Flowpath of aqueous phase:

1. Aqueous/organic segmented flow inlet
5. Exit of aqueous phase
6 Exit of any water droplets + some organic phase

The separator may also be configured to monitor the aqueous phase by connecting exit 5 to re-entry 7 and connection of exit 8 to the detector.

Exits 9 and 10 are for "waste" organic phase

Figure 3.1 Influence of mobile phase pH on the retention of some Group A compounds. Conditions: 5 µm Spherisorb-CN column (50 x 4.6 mm i.d.) with 0.05 M phosphate buffer-methanol-acetonitrile (90:5:5, v/v/v) at 30°C. Injection volume 10 µl, flow rate 1 $ml.min^{-1}$, monitored at 205 nm with 0.04 a.u.f.s.

Codes and Literature pKa for Group A Compounds

No	Compound	pKa
1	p-Hydroxynorephedrine	-
2	p-Hydroxyamphetamine	9.3
3	p-Hydroxymethylamphetamine	-
4	2-Phenylethylamine	9.83
5	Dexamphetamine	9.90
6	Phenmetrazine	8.40
7	Methylamphetamine	10.10
8	Mephentermine	10.40
9	Phendimetrazine	7.60
10	Chlorphentermine	9.60

Figure 3.25 Chromatograms of the highest (a) and lowest (b) concentrations for the calibration of p-hydroxynorephedrine (1), p-hydroxyamphetamine (2), p-hydroxymethylamphetamine (3), dexamphetamine (5), methylamphetamine (7). Injection volume (10 l) contained 10-80 $\mu g.ml^{-1}$. Conditions as in Fig. 3.23.

Figure 3.28 Chromatograms of the highest (a) and lowest (b) concentration for the calibration of p-hydroxynorephedrine (1), p-hydroxyamphetamine (2), p-hydroxymethylamphetamine (3), dexamphetamine 95) and methylamphetamine (7) (2-20 $\mu g.ml^{-1}$). Conditions as in Figure 3.23 (except for flow rate 0.5 ml/min).

Conditions for Figure 3.10(a)

5 - μm CPS - Hypersil column (50 x 4.6 mm.i.d) with 0.025 M Phosphate buffer (pH 7.2) - methanol-acetonitrile (90:5:5, V/V/V) at 30°C, 1 ml.min.$^{-1}$ flowrate monitored at 205 nm, 0.04 a.u.f.s.

Injection volume (10 μl) contained 6-12 ng of each solute.

Conditions for Figure 3.10(b)

Same as fig 3.10(a) but with a mobile phase of 0.02 M Phosphate buffer (pH 4.0) - propan-2-ol-acetonitrile (65:17.5:17.5, V/V/V).

Injection volume (10 μl) contained about 12 ng of each solute.

Figure 3.30(a) Chromatogram of the highest concentration (4.0 $\mu g.ml^{-1}$) for the calibration of Group B compounds. Conditions and solute code as in Figure 3.27.

Figure 3.14 Chromatogram of the separation of Group B compounds. Conditions: 5 μm Spheri-Cyano cartridge column (100 x 2.1 mm i.d.) with a mobile phase of 0.025 M phosphate buffer - propan-2-ol - acetonitrile (76: 12: 12, v/v/v) at 40°C. Injection volume (10 μl). Flow rate was 0.5 ml.min^{-1}, monitored at 205 nm with 0.04 a.u.f.s. Compounds as in Figure 3.10b.

Conditions for Figure 3.7

In all investigations 65% v/v 0.2 M phosphate buffer (pH 3.1) was used with:

A. 35% v/v Tetrahydrofuran (THF)

B. 35% v/v Propan-2-ol (Pr-2-OH)

C. 35% v/v Acetonitrile (ACN)

D. 17.5% : 17.5% v/v Pr-2-OH-ACN

E. 17.5%: 17.5% v/v THF-ACN

F. 17.5% : 17.5% v/v THF-Pr-2-OH

Figure 5.6.

Post-column conditions for the calibration of some Group A compounds.

Aqueous phase

a) stationary phase: Nucleosil SA 5 m 50 mm x 4.6 mm
mobile phase: 82.5% (v/v 0.2 M KH_2PO_4 (pH 5.0)
10% v/v methanol + 7.5% v/v acetonitrile.
Temperature: 57°C*
Flow rate: 1 ml/min
Injection volume: 10 µl

b)0.01M Sodium naphthalene-2-sulphonate
Flow rate: 0.30 ml/min

Organic phase

90% v/v chloroform + 10% v/v pentanol
Flow rate: 2.6 ml/min
max: 274 nm

* Aqueous phase was always cooled down from 57°C with ice crystals when using Nucleosil SA column.

Legend

1	p-Hydroxynorephedrine
2	p-Hydroxyamphetamine
3	p-Hydroxymethylamphetamine
5	Dexamphetamine
6	Phendimetrazine
7	Methylamphetamine
9	Mephentermine

Figure 5.10.

Post-column conditions for the calibration of some Group A compounds.

Aqueous phase

a) stationary phase: Hypersil SAS 100 x 2.1 mm
mobile phase: 90 % v/v 0.02 M phosphate buffer (pH 5.4)
5 % v/v methanol + 5 % v/v acetonitrile.
Temperature: 35°C
Flow rate: 0.5 ml/min
Injection volume: 10 µl

b) 0.01M Sodium naphthalene-2-sulphonate
Flow rate: 0.15 ml/min

Organic phase

90% v/v chloroform + 10% v/v pentanol
Flow rate: 1.30 ml/min
λ_{max}: 274 nm

Legend

4	2-Phenylethylamine
5	Dexamphetamine
6	Phendimetrazine
7	Methylamphetamine
8	Mephentermine
9	Phenmetrazine
10	Chlorphentermine

Figure 6.1 Chromatograms of separation of some Group A compounds of variable concentrations. UV detection at:

a) 205 nm

b) 254 nm

Other conditions as in Section 3.3.

CHROMATOGRAPHIC STUDIES ON THE DETECTION OF SOME BASIC DRUGS OF ABUSE

Thesis

Submitted by SHEWU OLADAPO BADIRU, M.Sc. (Pharm)

for the degree of Doctor of Philosophy

of the University of Bath

1989

Figure 6.2 Chromatograms of separation of some Group A compounds (variable concentrations).

a) Chromatography UV detection at 205 nm conditions as in Fig. 6.1.

b) Post-column UV detection at 274 nm conditions as in Fig. 5.6.